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**혈당변동이 췌장 베타세포의 GLP-1R 신호전달에 미치는
영향과 GLP-1R를 활성화시키는 약물의 항당뇨효과에 대
한 연구**

**Studies on the effect of glucose fluctuation on GLP-1R
signaling and anti-diabetic effect of drugs activating
GLP-1R in pancreatic beta cells**

2014년 8월

서울대학교 대학원

생명과학부

정 예 황

ABSTRACT

Diabetes mellitus is one of the most challenging and threatening worldwide health problems. The two most common forms of diabetes mellitus are type 1 and type 2 diabetes (T2DM) and about 90-95% of total diabetes cases have T2DM. Repeated fluctuations in plasma glucose are frequently observed in diabetic patients. Generally, fluctuations in glucose levels in T2DM patients were regarded as less important; however, recent studies have showed that glucose fluctuations are as important as average blood glucose levels, because glucose fluctuations aggravate β -cells function and increase the risk of diabetic cardiovascular complications more profoundly than sustained high glucose. Glucagon like peptide-1 receptor (GLP-1R) signaling in β -cells is a key molecular pathway, accounting for 60% of postprandial insulin secretion. However, GLP-1R signaling under glucose fluctuation conditions has not been determined.

In this study, it was hypothesized that glucose fluctuation conditions had more adverse effects on GLP-1-mediated cellular signaling in β -cells than sustained high glucose (SHG). To evaluate this hypothesis, I induced intermittent high glucose (IHG) conditions, mimicking glucose fluctuations, and SHG conditions in INS-1 cells, rat pancreatic β -cells. When INS-1 cells were exposed to IHG conditions, indeed, GLP-1R-mediated signaling, as evidenced by insulin secretion and calcium influx in β -cells, was reduced more severely than in SHG conditions. In IHG-treated INS-1 cells, ER-stress signaling was significantly higher than in SHG-treated cells, and GLP-1R expression was lower under IHG conditions than under SHG conditions. Taken together, these data suggest that

glucose fluctuation induce more severe impairment in GLP-1R signaling, via an increase in ER stress and a reduction in GLP-1R expression, and indicates the importance of tight regulation of blood glucose levels in T2DM patients.

Next, I investigated the postprandial glucose lowering and β -cells protective effect of exenatide, a GLP-1R agonist. When INS-1 cells were treated with exenatide, this agonist prevented β -cell death induced by high glucose, oxidative stress, and ER stress, which were induced more severely under glucose fluctuation conditions than SHG conditions. Additionally, after an oral glucose challenge in C57BL/6 mice, exenatide reduced blood glucose excursions effectively, without inducing hypoglycemia. Collectively, these data show that GLP-1R agonists can be efficacious in treating T2DM, reducing glucose fluctuation and β -cell damage.

Although exenatide has major effects on postprandial glucose lowering and β -cell survival, because of the inconvenience of subcutaneous injection twice per day, other approaches to activate GLP-1R (especially orally active GLP-1R agonists and DPP 4 inhibitors) have been sought. Historically, the discovery of orally active GLP-1R agonists has been unsuccessful, although recently, two large pharmaceutical companies have reported separately two small GLP-1R agonists, compounds 2 and B, showing the possibility of developing orally active GLP-1R agonists. Furthermore, because these two compounds have very different chemical structures, it is possible that they activate GLP-1R in different ways. However, there has been no report yet comparing the mechanisms of action between these compounds. To examine the possibly different mechanisms of action, first, compound 2 or compound B were co-treated with known peptide agonists or

antagonists and calcium influx was measured in CHO cells overexpressing GLP-1R. According to these data, I found three mechanisms of action between the two compounds: 1) compound 2 increased the responses to endogenous or exogenous peptide agonists, with no change in maximum efficacy. However, compound B increased both the responses and maximum efficacy. 2) Peptide antagonists augmented the response of compound 2, but did not alter compound B activity. 3) Compound 2 increased calcium influx consistently, while compound B did not. Intriguingly, when compound 2 and compound B were both added to CHO cells expressing hGLP-1R, compound B increased the activation responses of compound 2 additively, indicating that the compounds likely bind to different GLP-1R sites. Together, these results suggest that although two small-molecule agonists have been found to have agonist activity at GLP-1R, compounds 2 and B have different modes of action in activating GLP-1R, with different binding sites on GLP-1R. These results may also offer insight in understanding the as-yet unknown GLP-1R structure and in designing and discovering further novel orally active GLP-1R agonists.

Next, I characterized the mode of action and anti-diabetic effects of DA-1229, a novel DPP 4 inhibitor. DA-1229 was shown to be a potent and selective DPP-4 inhibitor in *in vitro* assays. After an oral glucose challenge in normal mice, DA-1229 increased GLP-1 and insulin levels, reducing blood glucose excursions. Additionally, DA-1229 improved insulin sensitivity in HF-DIO mice and delayed the onset of diabetes in young db/db mice. Collectively, these results suggest that DA-1229, novel DPP-4 inhibitor activating GLP-1R indirectly, may be a useful

substitute for exenatide and a new pharmacological choice for preventing and treating T2DM.

Key words: type 2 diabetes, glucose fluctuation, GLP-1R, ER stress, exenatide, compound 2, compound B, DPP 4 inhibitor, DA-1229

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CHAPTER 1.

INTRODUCTION

INTRODUCTION

1) Type 2 diabetes

Diabetes mellitus is one of the most challenging and threatening worldwide health problems. Although there are many studies on the molecular mechanisms of diabetes and anti-diabetic medications, the overall prevalence of diabetes is expected to double in the next 20 years and has been estimated to increase to 380 million by 2025 year (Lin and Zhongjie, 2010; Zimmet et al., 2001). If this estimation realizes, it is predicted that healthcare costs directly associated with diabetes will increase up to 13% of the global healthcare budget (Koliaki and Doupis, 2011).

The two most common forms of diabetes mellitus are type 1 and type 2 diabetes (T2DM). Both have similar symptoms: hyperglycemia, excessive urine production, compensatory thirst, increased fluid intake, blurred vision, unexplained weight loss, lethargy, and changes in energy metabolism (Lin and Sun, 2010). However, type 1 diabetes results from autoimmune-mediated destruction of pancreatic β -cells, causing an absolute deficiency of insulin, whereas T2DM is caused by defective insulin secretion and/or insulin action (Das and Elbein, 2006). About 90-95% of total diabetes cases have T2DM (Lin and Sun, 2010).

Type 2 diabetes mellitus (T2DM) is a complex, progressive, heterogeneous, and multisystemic diseases characterized by hyperglycemia because of impairments in insulin action in peripheral tissue, such as muscle, liver, and adipocyte, and/or insulin secretion in pancreatic β -cells (Lin and Sun, 2010). Regardless of blood glucose levels, insulin is synthesized in the β -cells constantly, stored within

vacuoles, and released by an increase of the blood glucose concentration. Insulin is a major hormone that regulates uptake of glucose from the blood into skeletal muscle cells and adipocytes, and insulin is also the principal signal to convert glucose into glycogen for internal storage in liver and skeletal muscle cells (Lin and Sun, 2010).

Insulin synthesis in β -cells is tightly regulated and varies greatly, with high glucose inducing insulin synthesis by 10-fold (Cnop et al., 2011). This imposes fluctuating demands on β -cells function, but sustained glucose fluctuation and insulin resistance lead β -cells, especially endoplasmic reticulum (ER), to synthesize and fold insulin beyond their capacity, and thereby inducing the unfolded protein response (UPR), a cellular stress response to adapt signaling pathway, to promote cell survival upon accumulation of unfolded or misfolded protein in the ER (Kaufman et al., 2010). In contrast to acute adaptive UPR activation, chronic activation increases expression of specific signal molecules, including proapoptotic transcription factor CAAT/enhancer-binding protein homologous protein (CHOP), and eventually results in β -cells death (Kaufman et al., 2010).

1) Glucose fluctuation in patients with type 2 diabetes

Repeated glucose fluctuation is a common phenomenon in patients with T2DM because their plasma glucose concentrations change frequently and markedly within a single day, whereas glucose concentrations are tightly regulated in normal subjects (Mazze et al., 2008). The results of several clinical and preclinical studies have shown that diabetic cardiovascular complications are

increased more profoundly as the result of postprandial glucose or glucose fluctuation condition, rather than the average blood glucose level, as indicated by hemoglobin A1c (HbA1c) levels (Gerich, 2003; Ceriello et al., 2004; Heine et al., 2004; Meigs et al., 2002). The Diabetes Control and Complication Trial (DCCT) study showed that the development and progression of microvascular complications were affected markedly by the extent of blood glucose excursion, postprandial glucose level (Kim et al., 2010). More specifically, another clinical study indicated that the production of free radical was associated with glucose fluctuation assessed by the mean amplitude of glycemic excursion (MAGE), but rather with average blood glucose (Monnier et al., 2006). In *in vitro* studies, it has been reported that intermittent hyperglycemia, mimicking glucose fluctuation situations, led to more adverse effects on the survival and function of vascular endothelial cells through induction of reactive oxygen species (ROS), as compared with chronic hyperglycemia (Quagliaro et al., 2003; Monnier et al., 2006; Ceriello et al., 2008).

According to recent studies, glucose fluctuation also affects β -cells function negatively and induces β -cells damage. Hou et al. showed that intermittent high glucose (IHG) conditions induced a higher degree of ER stress and oxidative stress in INS-1 cells as compared to sustained high glucose (SHG) conditions. In addition, IHG conditions, compared to SHG conditions, induced more serious impairments of insulin release in response to high glucose levels in rat islets and INS-1 cells (Hou et al., 2008). Kim et al. demonstrated that glucose fluctuation also induced apoptosis and dysfunction of INS-1 cells more than sustained high glucose, through induction of the expression of Mn-SOD and Bcl-2, apoptotic marker genes, and

deacetylation of FOXO (Kim et al., 2010; Kim et al., 2012). However, the effect of glucose fluctuation in β -cells has not been fully established.

2) *GLP-1R signaling*

Glucagon-like peptide-1 (GLP-1) is an insulinotropic incretin hormone (Orskov et al., 1994). GLP-1 is secreted from gut endocrine L-cells by meals and nutrients. The releasing pattern of GLP-1 is biphasic: the first phase occurring at 10-15 min after oral injection and the second phase occurring at 30-60 min (Lee and Jun, 2014). Since majority of L cells are located in the distal region of the small intestine, the first secretion of GLP-1 seems to be regulated by neurotransmitters and the vagus nerve rather than by direct activation of L cells (Rocca and Brubaker, 1999). The molecular signaling for GLP-1 secretion involves glucose-sensing signals including glucokinase, ATP-dependent potassium channel, and sodium glucose cotransporter-1 (Gribble et al., 2003). GPRs (GPR119, GPR40, and GPR120), expressed in the intestine, are also involved in GLP-1 secretion (Whalley et al., 2011).

The GLP-1 (1-37) precursor peptide is modified to the major active circulating form, GLP-1 (7-36) amide, by post-translational modification in L-cells (Orskov et al., 1994). Circulating GLP-1(7-36) amide is rapidly cleaved by the DPP-4 to GLP-1 (9-36), causing a GLP-1 half-life of about 2 min. The circulating GLP-1 is finally eliminated through renal and hepatic clearance (Lee and Jun, 2014).

GLP-1 signaling is started by binding to the membrane-bound GLP-1R. The GLP-1R is a ligand-specific and one of the guanine nucleotide-binding protein-

coupled receptor (GPCR) family B with 7 transmembrane domains and a long extracellular loop domain (Drucker et al., 1987; Thorens, 1992). The human GLP-1R gene is localized on the long arm of chromosome 6p21 and encodes a 463 aminoacids or 64-kDa protein (Stoffel et al., 1993). GLP-1R is expressed in various tissues including pancreatic β -cells, pancreatic ducts, kidney, lung, heart, skin, immune cells, hypothalamus, hippocampus, and cortex. Activated GLP-1R by GLP-1 leads to lots of beneficial effects for treatment of diabetes: increasing insulin secretion, delaying gastric emptying, inhibition of food intake, and improving insulin sensitivity (Fig. 1) (Lee and Jun, 2014).

From among lots of function of GLP-1, major GLP-1's function is to increase insulin secretion in β -cells. Glucose-stimulated insulin levels were reduced by about 60% in GLP-1R knockout mice, compared to normal mice, thereby resulting in higher fasting blood glucose levels and impaired glucose tolerance after oral or intraperitoneal administration of glucose (Scrocchi et al., 1996). Some clinical study also shows that GLP-1 approximately accounts for 70% of the total insulin secretion after the administration of oral glucose (Lee and Jun, 2014).

GLP-1-induced insulin secretion is mainly regulated by two separate pathways: cAMP-PKA and cAMP-Epac2 signaling pathways. The activated GLP-1R through binding of GLP-1 causes sequences of events: activation of adenylate cyclase, production of cAMP, activation of Protein kinase A (PKA) and Guanine nucleotide exchange protein activated by cAMP (Epac), inhibition of potassium-ATP channels, increase of intracellular Ca^{2+} levels, and stimulation of exocytosis of insulin secretory granules (Fig. 2) (Lee and Jun, 2014). Simultaneously, glucose is converted into the pyruvate through glycolysis, and the pyruvate is moved to the

mitochondria and metabolized through the tricarboxylic acid (TCA) cycle, causing an increase of ATP-to-ADP ratio and a closure of the ATP-sensitive potassium channels (K_{ATP}^+ channels) (Rondas et al., 2013).

In addition to the acute effect of GLP-1 on insulin secretion, GLP-1 also increases insulin gene expression (Wang et al., 1995). GLP-1 induces insulin gene transcription through cAMP/PKA-dependent and -independent signaling pathways and Ca^{2+} signaling pathways (Lee and Jun, 2014). Important genes in this signaling are transcription factors like pancreatic and duodenal homeobox 1 (Pdx-1), cAMP response element-binding protein (CREB), and nuclear factor of activated T-cells (NFAT) (Lawrence et al., 2002; Wang et al., 2005). GLP-1 activates EGFR which results in activation of PI3-kinase/PKB pathway, increase of phosphorylation and nuclear exclusion of FoxO1, consequently inducing FoxA2 binding to the Pdx-1 gene promoter and increasing Pdx-1 gene expression (Buteau et al., 2006). Pdx-1 binds insulin gene and increases insulin gene expression (Buteau et al., 2006). Activated CREB also contributes to the increase of insulin mRNA expression (Skoglund et al., 2000). In addition, increased Ca^{2+} activates calcineurin, inducing the nuclear localization of NFAT and activating the transcription of several genes involved in insulin production (Buteau et al., 2006).

GLP-1 also acts as an anti-apoptotic and/or proliferative agent in β -cells. When GLP-1R agonists were treated in purified rat β -cells or human islets, cytokine-induced and high glucose-induced apoptosis were reduced (Li et al., 2003; Buteau et al., 2004). In addition, the GLP-1R agonists prevented β -cells apoptosis in diabetic model animal: Zucker diabetic rats, db/db mice, and streptozotocin (STZ)-induced diabetic mice. In these animal models, consequently, glucose-

stimulated insulin secretion response and β -cells mass were restored by GLP-1R agonists (Doyle and Egan, 2007).

A variety of transcription factors and signaling molecules has effects on GLP-1 induced β -cell anti-apoptosis and proliferation. These signaling pathways are mediated by PI3K/PKB and cAMP/PKA/CREB pathway (Lee and Jun, 2014). Exenatide treatment in INS-1 cells and human pancreatic cells increase the expression of Pdx-1, and in islets of β -cell-specific pdx-1-deficient mice, exenatide fails to increase or recover the β -cell proliferation (Movassat et al., 2002; Li et al., 2005). In addition, IRS2 is implicated for GLP-1R-mediated β -cell proliferation, and insulin-like growth factor-1 receptor signaling pathways stimulated by GLP-1 is needed for the regulation of β -cell proliferation (Cornu et al., 2010). In INS-1 cells, GLP-1 treatment induces the expression of cyclin D, involving PKA, PI3 kinase and MEK/ERK signaling pathways. PI3K/PKB signaling and cAMP/PKA/CREB-mediated cyclin D1 expression also contribute to the protective effect of exendin-4 (Friedrichsen et al., 2006).

3) *Small GLP-1R agonists*

Active GLP-1 is rapidly degraded by dipeptidyl peptidase-4 (DPP-4) in vivo, which removes the two N-terminal GLP-1 amino acid residues and generates the biologically inactive form, GLP-1 (9-36) (Cho et al., 2006). For this reason, GLP-1 has presented considerable difficulties for therapeutic use; thus, longer-acting derivatives of GLP-1, such as exenatide and liraglutide, have been developed (Ogata et al., 2007; Schisano et al., 2011; Piconi et al., 2006). Exenatide, a 50% sequence homologue of native GLP-1, activates GLP-1R more potently than GLP-

1 and has been approved to treat type 2 diabetes. Although GLP-1 and its derivatives have therapeutic potential, these molecules must be administered by injection; thus, many researchers are attempting to discover orally active drugs activating GLP-1R: Small-molecule GLP-1R agonists and DPP4 inhibitors (Hou et al., 2008).

The GLP-1 receptor is one of the B/II family of seven transmembrane G protein-coupled receptors (GPCRs) and the class also includes receptors for peptide hormones such as secretin, GLP-1, glucose-dependent insulintropic polypeptide (GIP), glucagon, vasoactive intestinal peptide (VIP), corticotropin-releasing factor (CRF), calcitonin, and parathyroid hormone (PTH) (Laburthe et al., 1996; Fredriksson et al., 2003; Lagerstrom et al., 2008). The discovery and development of non-peptide and orally active agonists of these receptors has been basically failed (Sloop et al., 2010). This difficulty has contributed to the mechanisms of recognizing ligands and activating signaling used by class B GPCRs mechanistically different compared to class A GPCRs, for which many drug-like small molecules have been developed (Sloop et al., 2010). Specifically, peptide endogenous ligands of class B GPCRs bind to the large extracellular domain (ECD) of the receptors and initiate signaling, but the ligands of class A receptor primarily interact with residues located within the membrane spanning α -helical regions (Laburthe et al., 2007). This extracellular domain of class B receptors makes contacts with COOH-terminal residues of the ligands and positions the NH₂-terminus of the ligand to interact with important determinants in the transmembrane regions of receptors, therefore activating heterotrimeric G-proteins and subsequently signaling molecules (Castro et al., 2005; Hoare et al., 2005).

The recent reports describing GLP-1 receptor activation by a series of small molecules of substituted quinoxalines, cyclobutanes, and pyrimidines derivatives suggest that it be possible to discover and develop non-peptide and orally active GLP-1 receptor agonists (Sloop et al., 2010). From among those, compound 2 (6,7-dichloro-2-methylsulfonyl-2-N-tert-butylaminoquinoxaline) and compound B (4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine) are ones of the promising small ago-allosteric modulators of GLP-1R, which have more drug-like properties (Fig. 3) (Knudsen et al., 2007; Teng et al., 2007; Sloop et al., 2010). Both compound 2 and compound B increase cAMP by activating GLP-1R and increase insulin secretion *in vivo* or *ex vivo*. Coopman et al. reported that although compound 2 and GLP-1 have comparable GLP-1R activation effects, these molecules have different molecular signaling, such as calcium influx response and receptor internalization (Coopman et al., 2010). However, the modes of action of compound 2 and compound B have not been compared directly.

4) DPP-4 inhibitors

DPP-4 is about 110-150 kD per subunit, and exists as a membrane-bound form or soluble circulating form in plasma. DPP-4 is widely distributed in numerous tissues including T-cells, B-cells and natural killer cells. The enzymes, fibroblast activation protein (FAP or DPP 5), DPP8 and DPP9, and the catalytically inactive proteins, DPP6 and DPP 10, are related to DPP-4 structurally (Demuth et al., 2005). The endogenous substrates of DPP-4 are chemokines, cytokines, endomorphines, hormones of the pancreatic polypeptide family and almost all peptides of the PACAP/glucagon peptide family, including GLP-1 and GIP (Kieffer

et al., 1999). Since DPP-4 has no tissue-specific activity differences, DPP-4 has specific substrate, and DPP-4 is not controlled by limited proteolysis and by endogenous inhibitors, DPP-4 inhibitors should have therapeutic potential in different pathophysiological processes, and one of pivotal and successful approaches is to develop the DPP-4 inhibitors for the treatment of Type 2 diabetes (Demuth et al., 2005).

DPP-4 inhibitors are small molecular-weight and orally administrated drugs that inhibit > 90% of DPP-4 activity. These inhibitors prevent the rapid degradation of GLP-1, thereby improving glycemic control by preventing the rapid degradation of GLP-1 and increasing of active GLP-1 level (Brunton, 2014). Currently, there are three Food and Drug Administration (FDA)-approved DPP-4 inhibitors: sitagliptin, saxagliptin and linagliptin. In the clinical trials, DPP-4 inhibitors effectively lower fasting and postprandial plasma glucose concentration. In addition, comparable HbA1c reductions of 0.4-0.7% have been reported with these inhibitors mono-therapy for 26 weeks (Gerich, 2010). In preclinical studies, DPP-4 inhibitors protect pancreatic β -cells from the enhanced apoptosis, promote β -cells proliferation and neogenesis (Butler et al., 2003). Data from clinical studies in T2DM patients, markers of β -cells function, the homeostasis model assessment of β -cells function (HOMA-B), is declined with placebo, but improved with alogliptin (Gerich, 2010). However, the relatively modest physiological levels of GLP-1 achieved with DPP-4 inhibitors may lead to the neutral effect on body weight unlike exenatide, a GLP-1R agonist (Brunton, 2014). No hypoglycaemia was reported with any DPP-4 inhibitors in clinical trials, and common adverse events were mild-to-moderate gastrointestinal

complaints (nausea, vomiting, diarrhoea) (Brunton, 2014).

DA-1229, (R)-4-[(R)-3-amino-4-(2,4,5-trifluorophenyl)-butanoyl]-3-(*t*-butoxymethyl)-piperazin-2-one, a novel and orally available DPP-4 inhibitor under development to improve glycemic control in type 2 diabetic patients, was synthesized in an effort to search for more potent and selective DPP-4 inhibitors (Fig. 3) (Kim et al., 2011). In this study, to ascertain its therapeutic potential, I investigated the pharmacodynamic profile of DA-1229 and effects of DA-1229 on insulin resistance and development of diabetes in HF and db/db mice.

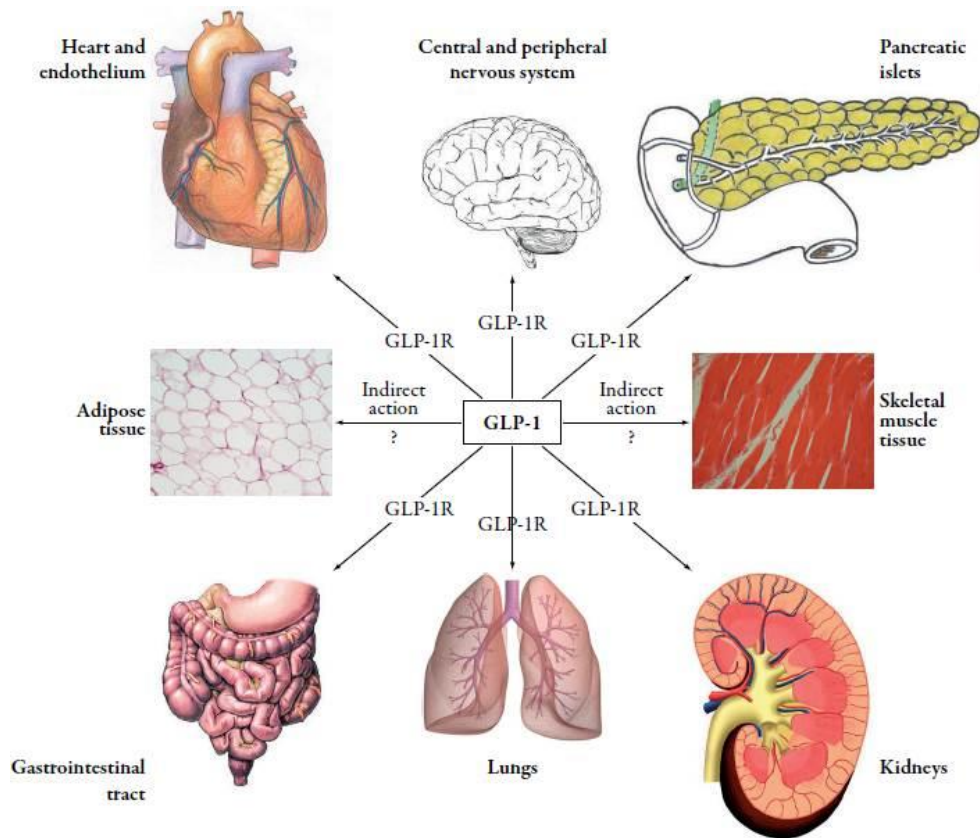


Figure 1.Major sites of GLP-1 action in the human body (Koliaki and Doupis, 2011)

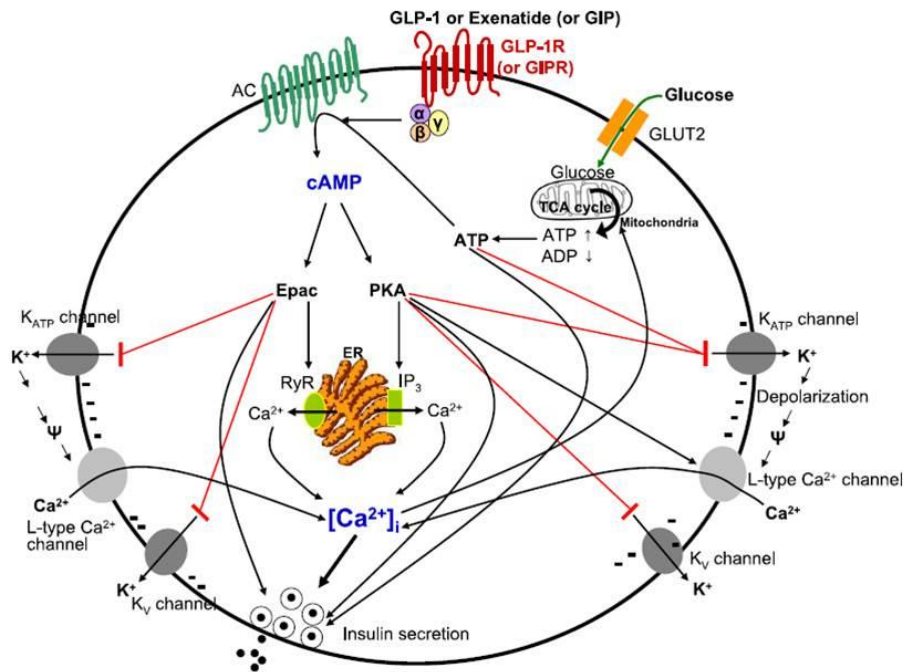


Figure 2. A schematic representation of the main molecular events during incretin-induced insulin secretion from β -cells (Kim and Egan, 2008).

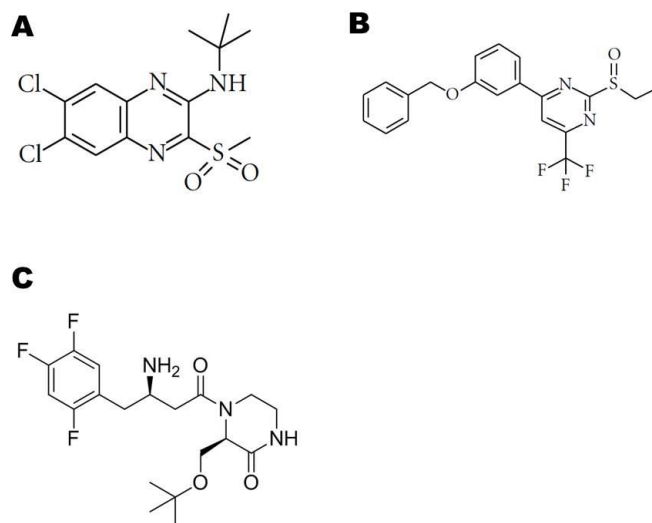


Figure 3. Chemical structures of compound 2, compound B, and DA-1229.

(A) compound 2 (6,7-dichloro-2-methylsulfonyl-2-N-tert-butylaminoquinoxaline) (Knudsen et al., 2007), (B) compound B (4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine). (Sloop et al., 2008) DA-1229 ((R)-4-[(R)-3-amino-4-(2,4,5-trifluorophenyl)butanoyl]-3-(t-butoxymethyl)piperazin-2-one) (Kim et al., 2008)

PURPOSE

In chapter 2, first, I examined the effects of glucose fluctuations on GLP-1-related signaling in β -cells. Repeated fluctuation is a common phenomenon observed in patients with T2DM. Several recent studies have reported that glucose fluctuations induced more oxidative and endoplasmic reticulum stress in β -cells, leading to adverse effect on β -cell function. However, GLP-1R signaling under glucose fluctuation conditions has not been examined. Second, I investigated the blood glucose-lowering effect and β -cell-protecting effects of exenatide, a GLP-1R agonist. Based on these studies, I want to evaluate whether drugs activating GLP-1R may be useful drugs for treating T2DM patients with high glucose fluctuations.

In chapter 3, I investigated the different modes of action and anti-diabetic effects of drugs activating GLP-1R directly versus indirectly. First, I compare the mode of action of two GLP-1R agonists (compounds B and 2) directly. Historically, the discovery of orally active and small-molecule agonists of GLP-1R has been unsuccessful. Recent reports on these two compounds suggest that it may be possible to develop orally active GLP-1 receptor agonists. However, the differences in the modes of action of these compounds have been not studied. Second, I investigate the *in vitro* and *in vivo* profiles of DA-1229, a novel dipeptidyl peptidase-4 (DPP-4) inhibitor. Additionally, I examine the effects of DA-1229 on insulin resistance and the development of diabetes in HF and db/db mice to assess its therapeutic potential.

CHAPTER 2.

Effect of glucose fluctuation on GLP-1R signaling in β -cells and anti-diabetic effects of exenatide

Section 1.

Effects of glucose fluctuation on GLP-1R signaling in β -cells

INTRODUCTION

Hyperglycemia is closely related to β -cells dysfunction and known to be a critical cause of type 2 diabetes (Gerich, 2003). An overwhelming body of evidence suggests that glucagon-like peptide-1 (GLP-1), an incretin hormone that stimulates insulin secretion, is modestly reduced in type 2 diabetes (Legakis et al., 2003). The subsequent actions of GLP-1 are mediated by its specific receptor, GLP-1R, thereby stimulating the adenylyl cyclase pathway, which induces increases in intracellular cAMP and calcium influx (Yada et al., 1993; Drucker, 2006). Via this important signaling pathway, GLP-1 exerts its insulin-releasing effects in response to glucose in pancreatic β -cells (Girard, 2008).

Chronically elevated glucose concentrations result in glucose toxicity and ultimately β -cells death (Favaro et al., 2008). Recently, several studies have demonstrated that repeated fluctuation from normal to high glucose concentrations, in addition to chronic and persistent hyperglycemia, is another important phenomenon inherent to type 2 diabetes (Cho et al., 2006; Ogata et al., 2007). Accumulated data appear to demonstrate that fluctuating high glucose levels influence the development of diabetic complications via the generation of reactive oxygen species (Schisano et al., 2011; Piconi et al., 2006). Other recent studies indicated that fluctuating high glucose levels affect β -cell functions and induce cell death (Hou et al., 2008; Kim et al., 2010). Hou et al. showed that intermittent high glucose (IHG) conditions induced higher degree of endoplasmic reticulum (ER) and oxidative stress in INS-1 cells as compared to sustained high glucose (SHG) conditions (Hou et al., 2008). In addition, IHG conditions, compared to SHG

conditions, induce a more serious impairment of insulin release in response to high glucose levels in rat islets and INS-1 cells (Hou et al., 2008). Under SHG conditions, GLP-1R expression is downregulated, which contributes to the impaired incretin effects found in diabetes (Xu et al., 2007; Pan et al., 2009). On the other hand, although IHG conditions seriously affect type 2 diabetic patients, the effects of IHG on the subsequent signaling of GLP-1 have not been elucidated clearly.

Therefore, in order to evaluate the effects of IHG on GLP-1-related signaling and β -cell functions, I measured insulin secretion and calcium influx in response to GLP-1, as well as the expression of GLP-1R in INS-1 cells under SHG or IHG conditions.

EXPERIMENTAL PROCEDURE

1. Cell cultures and treatment conditions

INS-1 cells (rat insulinoma cell line, passages 10–20) were cultured in RPMI1640 medium supplemented with 11 mM glucose, 10% fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES pH 7.4, 50 μ M 2-mercaptoethanol, 100 U/mL penicillin G, and 100 μ g/mL streptomycin at 37°C and 5% CO₂ (Asfari et al., 1992). Cells were seeded at 20,000 cells/well in 96-well multiplates in RPMI1640 medium. After 48 h, the cells were treated for 72 h with RPMI1640 medium containing 30 mM glucose to produce sustained hyperglycemic conditions. To produce IHG conditions, the cells were treated alternately (every 12 h) with media containing either 11.1 mM or 30 mM glucose. In the control and SHG cell samples, the media was also changed every 12 h.

2. Determination of insulin secretion and cellular insulin content

After 72 h of treatment, the INS-1 cells were rinsed twice in phosphate-buffered saline (PBS), then pre-incubated in Krebs-Ringer bicarbonate HEPES buffer (KRBH buffer: 11.5 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 1 mM MgCl₂, 25 mM HEPES pH 7.4, and 0.5% bovine serum albumin [BSA]) without glucose at 37°C. After 1 h, INS-1 cells were incubated for 30 min in fresh KRBH buffer supplemented with glucose and 1 nM GLP-1 at 37°C. The insulin secreted into the medium was determined with an insulin enzyme-linked immunosorbent assay (ELISA) kit (ALPCO, Windham, NH) in accordance with the manufacturer's instructions. Finally, the value of insulin was normalized to the total protein content

as measured via the bicinchoninic acid (BCA) method.

From the cell layer, insulin content was measured using the method described by Hamid et al. (Hamid et al., 2002). In brief, the cells were incubated in 200 μ L of acid-ethanol solution (1.5% (v/v) HCl, 75% (v/v) ethanol, 23.5% H₂O) at 4°C overnight. The insulin levels in the lysates were detected and normalized to the total protein content, as described above.

3. Polymerase chain reaction

After 72 h of exposure to high glucose conditions, total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's recommended protocols. First-strand DNA was synthesized using reverse transcriptase with random hexamers as primer. The amplification of the target gene was carried out using the following specific primers: binding protein (Bip) (forward, 5'-CCA CCA GGA TGC AGA CAT TG-3'; reverse, 5'-AGG GCC TCC ACT TCC ATA GA-3'), C/EBP homologous protein (CHOP) (5'-CCA GCA GAG GTC ACA AGC AC-3'; reverse, 5'-CGC ACT GAC CAC TCT GTT TC-3'), insulin (forward, 5'-TCT TCT ACA CAC CCA TGT CCC-3'; reverse, 5'-GGT GCA GCA CTG ATC CAC-3'), GLP-1R (forward, 5'-CCT GAG GAA CAG CTC CTG TC-3'; reverse, 5'-CAG TGA GGC CAG GAT AGA GC-3'), and β -actin (forward, 5'-GGC TGT GTT GTC CCT GTA TG-3'; reverse, 5'-AGG AAG GAA GGC TGG AAG AG-3'). Amplification cycles were optimized for each gene to be in linear range. The expression of target genes was normalized to that of β -actin.

4. Western blotting

After 72 h, the INS-1 cells were washed once with PBS. Then, proteins were extracted using RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany) and centrifuged at 4°C and $10,000 \times g$ for 10 min. Total protein concentration in the supernatant was quantified via the BCA method. Next, 20 µg total protein of each sample was separated on a 4%–12% Bis-Tris pre-cast gel (Invitrogen, Carlsbad, CA). The separated proteins were then transferred to PVDF membranes, followed by blocking with 5% skim milk in 0.1% TBST (TBS with 0.1% Tween-20). Then, the membranes were reacted with the primary antibodies for GLP-1R (1:1,000) or β -actin (1:3,000) at 4°C overnight, reacted sequentially with rabbit polyclonal horseradish peroxidase-conjugated secondary antibody (1:2,000 for GLP-1R and 1:5,000 for β -actin) at room temperature for 1 h, and developed via ECL chemiluminescence. The expression of GLP-1R was normalized that of β -actin.

5 Caspase 3/7 activity assay

The caspase 3/7 activity assay was conducted in accordance with the manufacturer's instructions (G8091, Promega, Madison, WI). In brief, after 72 h, INS-1 cells were treated with caspase-Glo3/7 reagent in 96-well plates. The plates were gently shaken for 30 s and incubated at room temperature for an additional 30 min. Luminescence was then measured with a luminometer (LmaxII384, Molecular Devices Inc., Sunnyvale, CA).

6. Calcium uptake assay

The calcium uptake assay was conducted using the FLIPR Calcium 5

assay in accordance with the manufacturer's instructions (R8185, Molecular Devices Inc.). In brief, after 72 h, the cells were loaded with $1 \times$ calcium dye in Hank's Balanced Salt Solution (HBSS; containing 5.4 mM KCl, 4.2 mM NaHCO₃, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 0.6 mM MgSO₄, 137 mM NaCl, pH 7.4) at 37°C. After 1 h, intracellular calcium changes were recorded at an excitation wavelength of 485 nm and emission wavelength of 525 nm at 1.3 s intervals for 150 s using a multireader Flexstation-3 (Molecular Devices Inc.). GLP-1 and glucose in the HBSS solution were earlier prepared in another plate and automatically applied to the cells after 20 s of recording.

7. Data analysis

Data are expressed as means \pm SEM. One-way ANOVA using SigmaStat 2.0 (SPSS, Chicago, IL) was used for comparisons among groups. When significant differences were detected, multiple comparisons were conducted via the Student-Newman-Keuls test.

3. RESULTS

1. Insulin secretion capacity by GLP-1 was reduced under IHG conditions in INS-1 cells

To examine the effect of IHG conditions on insulin secretion by GLP-1, I performed glucose-stimulated insulin secretion assays in INS-1 cells. At the end of 72 h of exposure, no significant differences were noted among the 3 groups (control, SHG, and IHG) in the presence of 3 mM glucose. When INS-1 cells were exposed to 11.1 mM glucose, the insulin release in the 2 hyperglycemia groups was significantly reduced as compared with the controls. Under these conditions, the level of insulin secretion was almost identical between the SHG and IHG groups. However, in the presence of 1 nM GLP-1, the level of insulin secretion was more profoundly decreased in INS-1 cells exposed to IHG (by $46.9 \pm 0.96\%$) compared to SHG (by $33.8 \pm 0.56\%$) (Fig. 4).

2. IHG conditions increased cellular toxicity with ER stress and reduced insulin expression in INS-1 cells

To evaluate the cytotoxic effects of IHG, I subsequently assessed caspase 3/7 activity in INS-1 cells. After 72 h of exposure, the activity of caspase 3/7 increased under SHG conditions as compared with controls and this increase was even more marked under IHG conditions (Fig. 5B). To further assess the toxic effects of IHG, I used reverse transcription-polymerase chain reaction (RT-PCR) to evaluate the expression of representative ER stress markers, especially Bip and CHOP. Exposure of INS-1 cells to SHG upregulated the expression of Bip by $1.3 \pm$

0.05 fold and CHOP by 1.2 ± 0.08 fold over controls ($P < 0.05$). IHG induced excessive activations of these 2 components of ER stress by 1.5 ± 0.06 fold and 1.4 ± 0.15 fold, respectively (Fig. 5A). Under SHG conditions, the insulin content in INS-1 cells was reduced by $38.1 \pm 1.7\%$ as compared with controls; however, there was no statistical significance as compared with IHG conditions (Fig. 6A). With regard to the insulin expression level, although it appeared that IHG induced a more severe reduction than SHG, no statistically significant differences were noted between the hyperglycemia groups (Fig. 6B).

3. Calcium influx in response to GLP-1 was decreased in INS-1 by IHG

Activated GLP-1R stimulates glucose-induced insulin secretion, which is known to be involved in the increase in intracellular calcium influx (Yada et al., 1993). To evaluate whether glucose fluctuation affects calcium influx, I assessed calcium influx capacity in INS-1 cells in response to 1 nM GLP-1 under control, SHG, and IHG conditions. SHG reduced calcium influx levels by $30.2 \pm 6.32\%$ as compared with controls (Figs. 7A and 7B). IHG produced a significantly larger reduction in calcium influx levels (by $49.2 \pm 5.9\%$ vs. controls).

4. GLP-1R expression was downregulated under IHG conditions in INS-1 cells

High glucose concentrations result in the downregulation of GLP-1R expression (Xu et al., 2007; Pan et al., 2009). To investigate the evidence of reduction in GLP-1 capacity, particularly under IHG conditions, I evaluated the expression of GLP-1R using RT-PCR and western blot. Under SHG and IHG conditions, the levels of GLP-1R mRNA in INS-1 cells were reduced by $24.3 \pm 2.51\%$

and $38.6 \pm 5.52\%$, respectively ($P < 0.05$) (Fig. 8A). In addition, the protein level of GLP-1R in INS-1 cells exposed to IHG was significantly lower compared to that of cells exposed to SHG (Fig. 8B). These data indicate that glucose fluctuation induces a relatively more severe impairment of GLP-1R expression, which may result in reduced β -cell function.

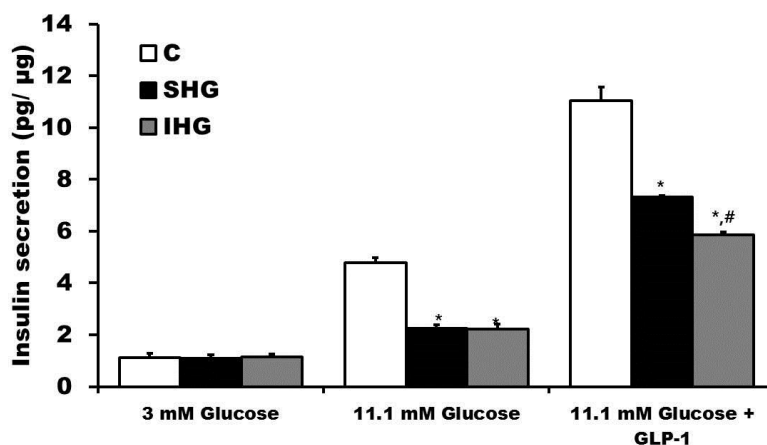


Figure 4. Insulin secretion response under SHG and IHG in INS-1 cells.

Following 3 days of exposure in the control, SHG, and IHG conditions, glucose-stimulated insulin secretion in INS-1 cells was measured with an ELISA kit and then calculated. Data are expressed as means \pm SEM of 3 separate experiments. *, $P < 0.05$ vs. C; #, $P < 0.05$ vs. SHG. Abbreviations: GLP-1, glucagon-like protein-1; C, control; SHG, sustained high glucose; IHG, intermittent high glucose.

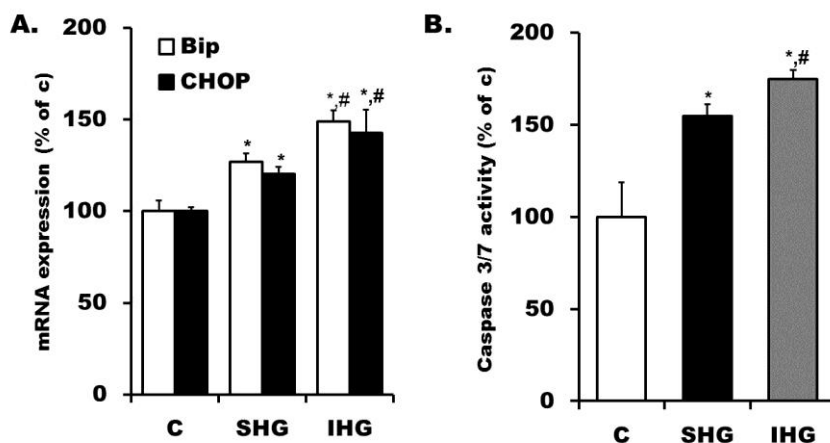


Figure 5. Caspase 3/7 activity and Bip and CHOP expression under SHG and IHG in INS-1 cells.

(A) After total RNA extraction and reverse transcription, *Bip* and *CHOP* expression were determined via PCR analysis. Data are expressed as the means \pm SEM of 3 separate experiments. (B) After the INS-1 cells were incubated for 72 h in the control, SHG, and IHG conditions, caspase 3/7 activity was assessed.

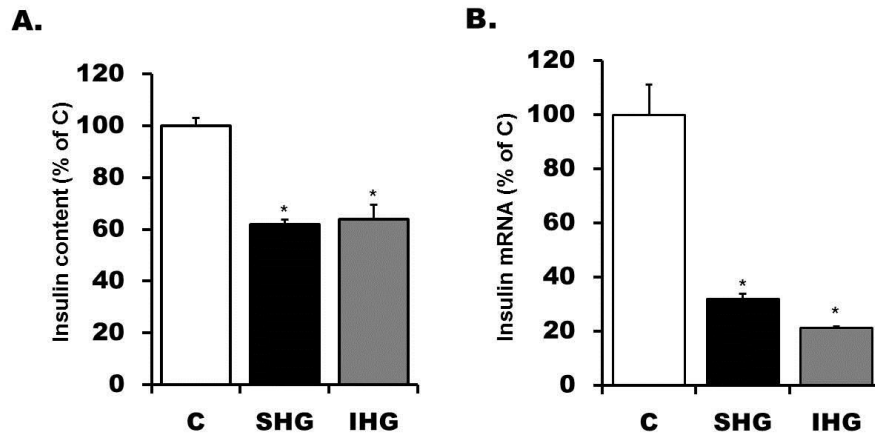


Figure 6. Expression of insulin under SHG and IHG in INS-1 cells.

(A) After total RNA extraction and reverse transcription, the gene expression of insulin was determined via PCR analysis. (B) Total insulin content in the INS-1 cells was extracted via the acid/ethanol method and detected with an ELISA kit. *, $P < 0.05$ vs. C; #, $P < 0.05$ vs. SHG.

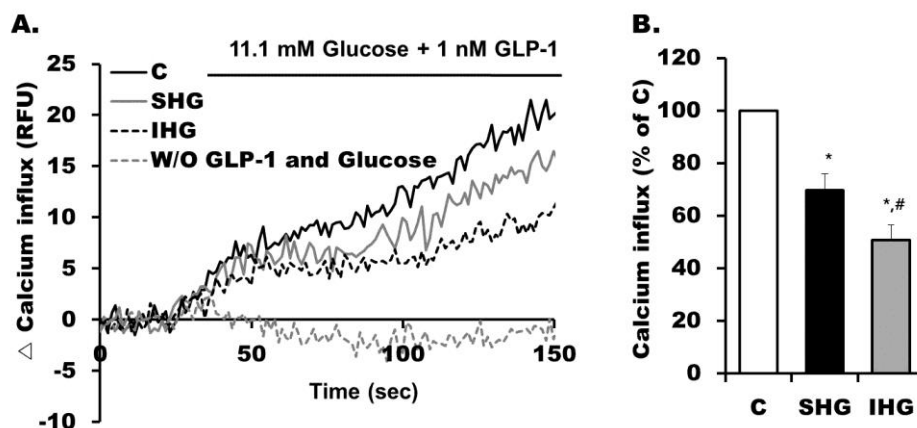


Figure 7. Calcium influx response under SHG and IHG in INS-1 cells.

(A) Calcium influx in INS-1 cells was assessed in the control, SHG, and IHG conditions using a FLIPR Calcium 5 assay. (B) Data are expressed as the means \pm SEM of 10 separate experiments. *, $P < 0.05$ vs. C; #, $P < 0.05$ vs. SHG.

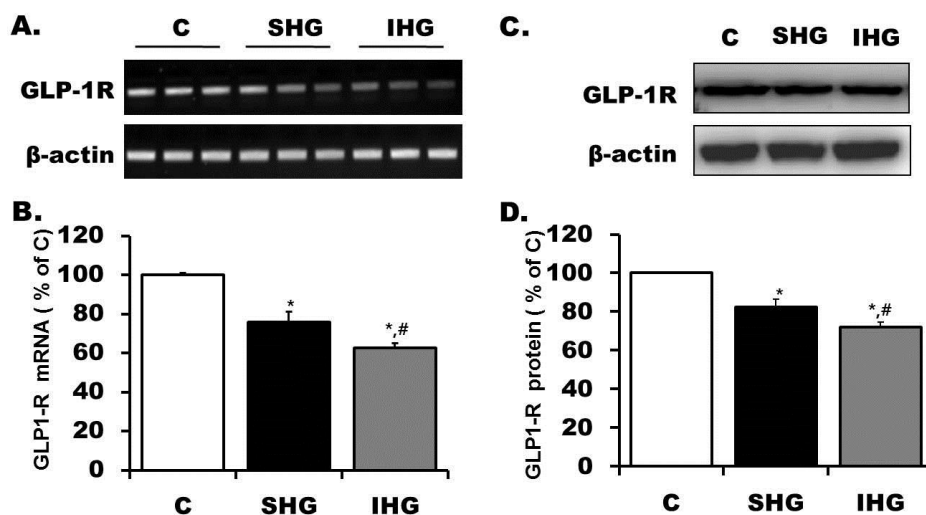


Figure 8. GLP-1R expression under SHG or IHG in INS-1 cells .

(A) The gene expression of GLP-1R was determined in the control, SHG, and IHG cell samples via RT-PCR. (C) Under the same conditions, GLP-1R expression was measured via western blot analysis. Densitometric analysis of (B) RT-PCR or (D) western blot is reported as the means \pm SEM of 6 separate experiments. *, $P < 0.05$ vs. C; #, $P < 0.05$ vs. SHG.

DISCUSSION

In this study, I showed that compared to SHG, IHG exposure of rat pancreatic INS-1 β -cells led to more marked reductions of subsequent GLP-1 signaling, as well as insulin secretion and calcium influx. Interestingly, the expression of GLP-1R at both the gene and protein levels was lower under IHG than SHG conditions.

Repeated glucose fluctuation is a common phenomenon in patients with diabetes because their plasma glucose concentrations change frequently and markedly within a single day, whereas glucose concentrations are tightly regulated in normal subjects (Mazze et al., 2008). The results of several clinical studies have shown that diabetic complications, especially cardiovascular disease, increased more profoundly as the result of postprandial glucose or peak glucose levels, rather than the average blood glucose level, as indicated by hemoglobin A1c (HbA1c) levels (Ceriello et al., 2004; Heine et al., 2004; Meigs et al., 2002). Furthermore, Kim et al. demonstrated that glucose fluctuation also induces apoptosis and dysfunction in INS-1 cells (Kim et al., 2010); thus, diabetic patients might experience more significant β -cell dysfunction under glucose fluctuation conditions than under chronic hyperglycemic conditions. However, the molecular changes associated with glucose fluctuation has not been thoroughly elucidated; therefore, studies of glucose fluctuation are clearly necessary to gain more insight into the complex phenomenon of diabetes.

An overwhelming body of evidence suggests that ER and oxidative stress inhibit gene transcription and/or translation (Shenton et al., 2006; Eizirik et al.,

2008). In addition, recent studies have shown that IHG is a more effective activator of ER and oxidative stress (Hou et al., 2008; Kim et al., 2010). In this study, I have confirmed that adverse ER stress increases more by glucose fluctuation than by sustained hyperglycemia, as result of higher expression levels of Bip and CHOP. Although I did not assess the changes in oxidative stress markers in this study, significant oxidative stress might also be induced under IHG conditions. Therefore, I suggest that reductions in GLP-1R expression might be affected by ER stress as well as oxidative stress. However, until now, the molecular mechanisms underlying the induction of increased adverse ER and oxidative stress in glucose fluctuation have not been clearly elucidated. One of the most likely explanations is that IHG conditions may not activate the same glucose toxicity regulators as SHG conditions (Hou et al., 2008). Another possibility is that the more prolonged epigenetic changes attributable to glucose variability also induce greater deteriorations and dysfunction in β -cells (Schisano et al., 2011). These speculations need to be elucidated in further investigations.

In the islets of pancreatectomized (Px) hyperglycemic and normal rats that were exposed to high glucose levels, GLP-1R expression as well as subsequent insulin secretion were also significantly reduced (Xu et al., 2007). In a previous study, GLP-1R knockout mice showed abnormalities in fasting glucose levels, glucose-dependent insulin secretion, and islet size (Hansotia et al., 2006). Therefore, in this study, lower GLP-1R expression as the result of glucose fluctuation might more significantly affect reductions of the responses mediated by GLP-1R activation. I anticipate that type 2 diabetic patients with frequent glucose fluctuation might have reduced GLP-1R expression as well as more severe

subsequent signaling in β -cells compared to chronic hyperglycemia patients. Furthermore, because GLP-1R is also expressed in other tissues such as the brain, liver, and heart (Ahren, 2004), glucose fluctuation can also affect the functions of GLP-1 in these tissues.

In addition to reductions in GLP-1R expression, chronic hyperglycemia also induces the desensitization of β -cells in response to glucose and, thereby, reduces glucose-stimulated insulin secretion (Yamazaki et al., 2000b). Tsuboi et al. demonstrated that INS-1 cells cultured in the presence of 30 mM glucose for 48 h showed reduced expressions of glucokinase and glucose transporter 2 (Glut2), which are glucose sensors in β -cells, leading to inhibition of glucose-stimulated insulin secretion (Tsuboi et al., 2006). In addition, the expression of synaptotagmin V, which regulates membrane vesicle fusion by sensing calcium levels, was reduced under hyperglycemic conditions and resulted in abnormal termination of vesicle exocytosis events (Tsuboi et al., 2006). Although the effects of repeated glucose fluctuation on those molecules have not been elucidated yet, it might be possible that decrease of glucose-related molecules were contributed to reduced cellular events in response to GLP-1 in IHG condition. Also, I supposed that the reduction in calcium uptake by IHG in this study was attributable to the diminution of this channel or calcium channel-activating molecules such as exchange protein activated cAMP (Epac) (Oestreich et al., 2009) as the result of ER and/or oxidative stress. Therefore, more reduced GLP-1-related subsequent events under IHG conditions than under SHG conditions might be attributable to lower GLP-1R expression and other mechanisms; further studies are necessary to evaluate the validity of this speculation.

Taken together, the results of this study demonstrated that glucose fluctuation triggers the reduction of GLP-1R expression and subsequent GLP-1 signaling in INS-1 cells, which may contribute to reductions in insulin secretion by GLP-1 to a greater degree than is observed in chronic hyperglycemia. From a clinical standpoint, our study suggests that strict regulation of the blood glucose concentration is necessary to prevent β -cell dysfunction mediated by the reduction of GLP-1R expression and subsequent GLP-1 signaling.

Section 2.

Anti-diabetic effects of GLP-1R

agonists, exenatide

INTRODUCTION

Repeated fluctuation in plasma glucose level is an important phenomenon frequently observed in diabetic patients. Recently, several studies have reported that glucose fluctuation, compared to chronic hyperglycemia, induced more severe ER-stress and oxidative stress in β -cells, thereby resulting in more adverse effects on β -cellsfunction (Hou et al., 2008; Kim et al., 2010).

Exenatide is a 39-amino acid peptide and a synthetic version of exendin-4 (Ex-4), a peptide isolated from the salivary secretions of Gila monster lizard, *Heloderma suspectum*. Exendin-4 shares 53% structural homology to human GLP-1. Since the lizard also synthesizes GLP-1, it is not lizard GLP-1 and is encoded by a different exendin-4 gene (Doyle and Egan, 2007). Exenatide is not degraded by DPP-4 because it contains histidine-glycine at its N-terminus, thereby having a relatively long half-life of 4-5 h. It binds and activates the GLP-1R with greater potency, compared to native GLP-1 (Doyle and Egan, 2007). Preclinical and clinical studies show that exenatide exerts a positive effect on insulin secretion, β -cells proliferation and survival (Dalle et al., 2013). However, the direct effects of exenatide under the glucose fluctuation condition have not been elucidated clearly.

EXPERIMENTAL PROCEDURE

1. Animals study

All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Research Center in Dong-A Pharm. Co., Ltd. Male C57BL/6J mice were obtained from Dae Han Biolink (Eumseong, Korea).

Six-week-old animals were acclimated for 1 week and assigned randomly to the corresponding groups (6 animals per group). The mice were fasted overnight and exenatide (0.3, 1, 3, 10 μ g/kg; suspended in 0.5% (w/v) methyl cellulose) was administered by subcutaneous injection 1 h before the oral glucose (2 g/kg) challenge. Blood glucose levels were measured at each indicated time point. The blood glucose excursions for 120 min are represented as the area under the curve (AUC_{0-90min}). Blood glucose AUC values of glucose-challenged groups were normalized by subtracting that of distilled water-challenged non-glucose control and then the percent inhibition values for each treatment were generated using the normalized AUC values over the vehicle-treated glucose control.

2. β -cells protection assay

For high glucose-induced β -cell death, INS-1 cells were co-treated with glucose (20, 30, or 40 mM) and each concentration of exenatide for 72 h. For STZ-induced β -cell death, serum-starved cells were pretreated with indicated concentrations of exenatide for 1 h, and then treated with 2 mM STZ dissolved in 0.1 M citrate buffer (pH 4.5), or with citrate buffer alone, in serum- and glucose-

free media for 1 h. The cells were then recovered in serum-free and 5.6 mM glucose-containing media and treated again with each drug for additional 17 h. In the case of thapsigargin which inhibits sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) and causes the depletion of ER calcium stores and activation of apoptosis (Tonnesen et al., 2009), the serum-starved cells were treated with 0.3 μM thapsigargin and each drug for 6 h in serum-free and 5.6 mM glucose-containing media. Finally, cell viability was assessed by determining cellular ATP levels. Briefly, 100 μl of reconstituted CellTiter-Glo™ reagent (Promega, Madison, WI, USA) was added to media, the plate was agitated for 2 min and ATP was measured using luminometer using a LmaxII384 (MDC, Sunnyvale, CA, USA). Cell viability was expressed as a percentage of control.

RESULTS

1. Exenatide lowers post prandial blood glucose effectively without inducing hypoglycemia issue.

The oral glucose tolerance test in C57BL/6 mice showed that, in a dose-dependent manner, the exenatide treatment significantly inhibited postprandial blood glucose excursions by 94.8 % (Fig. 9). Next I examined whether exenatide enable to induce hypoglycemia or not. First, in INS-1 cells, insulin secretion was increased by glucose in concentration dependent manner. When less than 5 mM glucose was treated in INS-1 cells, exenatide did not increase insulin secretion significantly, while a positive control, glimepiride, increased insulin secretion significantly (Fig. 10A). In C57BL/6 mice, when exenatide was treated without glucose loading, the blood glucose was lowered in dose-dependent manner, but the blood glucose was not reduced below 50 mg/dl (Fig. 10B). These data indicated that exenatide significantly reduced postprandial blood glucose excursions, but did not induce hypoglycemia.

3. Exenatide prevents high glucose, ER stress, and oxidative stress-induced INS-1 cells death.

INS-1 cells exposed to 20 mM glucose for 48 h did not exhibit any significant change in cell viability, whereas at higher concentrations, 40 mM, caspase 3/7 activity was significantly increased to 137.2% of that observed in control cells. When the incubation time was extended to 72 h, 20, 30, 40 mM glucose increased the caspase3/7 activity of INS-1 cells to 142.1, 135.6, or 123.9%

of that observed in unstimulated control cells, respectively (Fig. 11A). When both 40 mM glucose and exenatide were co-treated for 72 h in INS-1 cells, exenatide reduced the caspase 3/7 activity in dose dependent manner (Fig. 11B).

Glucose fluctuation induces more severe ER stress and oxidative stress in β -cells, compared to sustained high glucose (Hou et al., 2008; Kim et al., 2010). To evaluate the protection effect of exenatide against ER stress and oxidative stress, thapsigargin was treated to activate ER stress-induced cell death. When INS-1 cells were treated with 0.3 μ M thapsigargin for 6 h, their viability was reduced to 53.4%, while exenatide treatment completely blocked thapsigargin-induced cell death (Fig. 12). Next, serum starved INS-1 cells were treated with 2 mM STZ for 1 h to induce oxidative stress and recovered overnight in serum free media, and the viability decreased to 18.2% of control citrate buffer alone. However, when exenatide was treated during pre- and post-STZ treatments, cell viability increased in a dose-dependent manner. Maximum protective responses were obtained at doses of 10 nM exenatide (Fig. 13A and B).

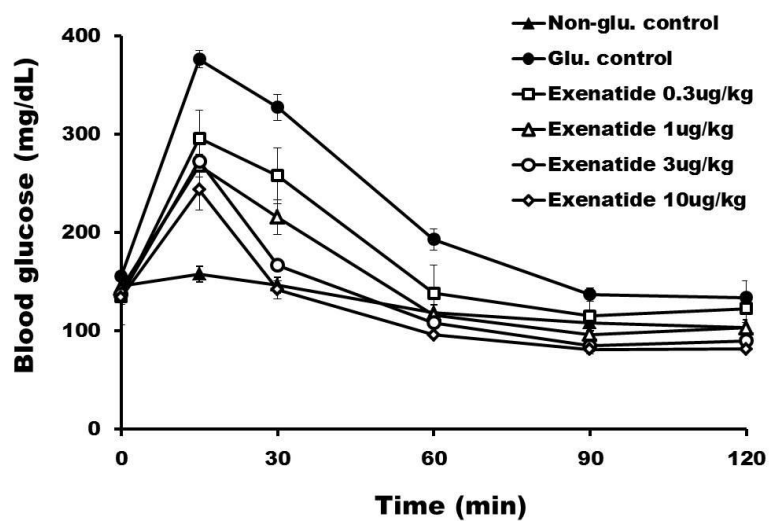


Figure 9. Effects of single dose of exenatide on the blood glucose excursion.

C57BL/6 mice were fasted overnight and administered a single dose of the vehicle alone (0.5% (w/v) methyl cellulose) or exenatide by a subcutaneous injection. At 30 min post-dose, the mice were given a glucose solution (2g/kg). After the glucose loading, blood glucose was measured at the indicated time points for 120 min

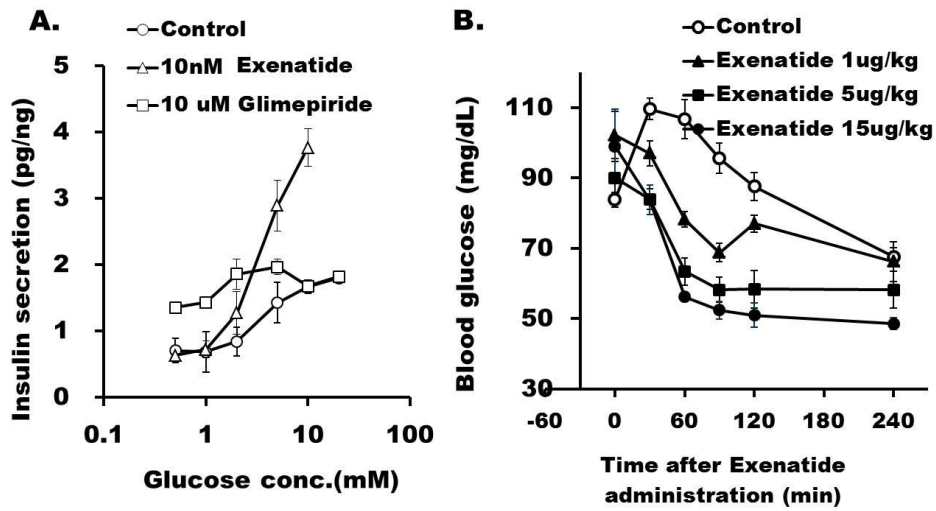


Figure 10. Effect of exenatide on inducing hypoglycemia.

(A) Glucose dependent response of insulin secretion in INS-1 cells. (B) C57BL/6 mice fasted 4 h before each concentration of exenatide were treated without glucose loading. Blood glucose was measured at the indicated time points for 240 min

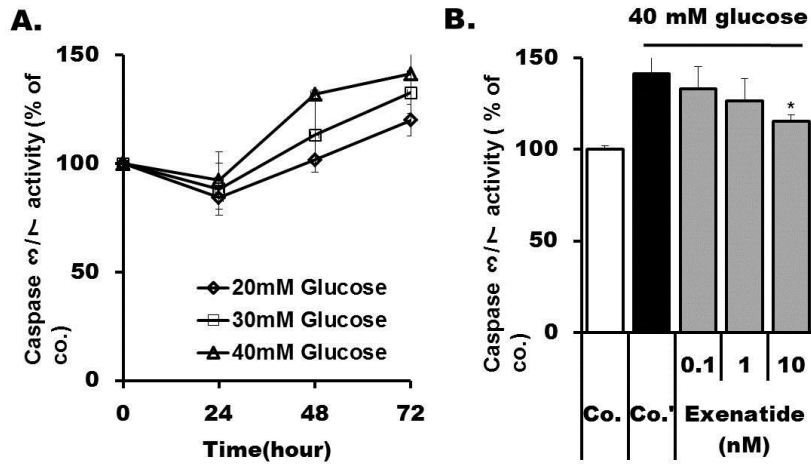


Figure 11. Effect of exenatide on reduction of high glucose-induced caspase 3/7.

(A) Time and glucose concentration dependence of Caspase 3/7 activity in INS-1 cells. (B) INS-1 cells were co-treated with 40 mM glucose and each concentration of exenatide. After 72 hr, caspase 3/7 activity was measured. Data from three individual experiments were presented as mean \pm S.E. *, $P < 0.05$ vs. co' (40 mM glucose treatment control).

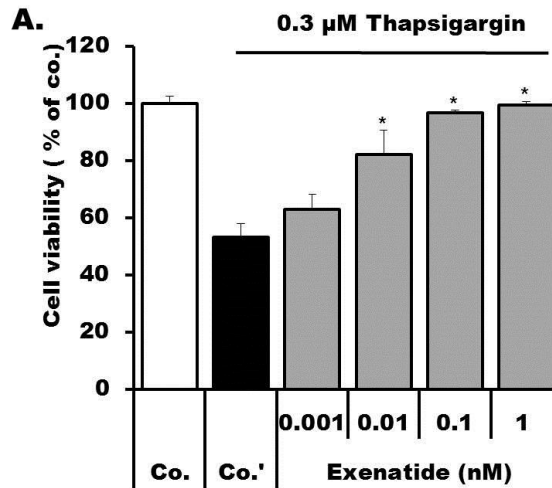


Figure 12.Effect of exenatide on decrease of thapsigargin-induced β -cell death.

INS-1 cells serum-starved overnight were treated in the absence or presence of exenatide, with or without 0.3 μ M thapsigargin for 6 h. At the end of each experiment, cell viability was assessed by cytosolic ATP levels and data from three individual experiments were presented as mean \pm S.E. *, $P < 0.05$ vs. control in the presence of 0.3 μ M thapsigargin.

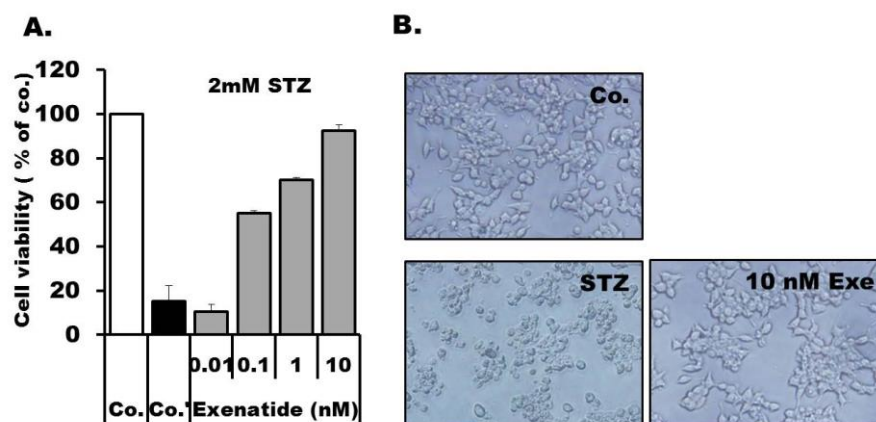


Figure 13. Effect of exenatide on prevention of STZ-induced β -cells death.

(A) INS-1 cells, serum-starved overnight, were pre- and post-treated with each drug with or without 2 mM STZ for 1 h. At the end of each experiment, cell viability was assessed by cytosolic ATP levels and data from three individual experiments were presented as mean \pm S.E. (B) representative cell image. *, $P < 0.05$ vs. control in the presence of 2 mM STZ.

DISCUSSION

Recent preclinical studies confirm that exenatide exerts a positive effect on insulin secretion, β -cell proliferation and survival (Dalle et al., 2013). However, the important question remaining unanswered in the research is whether GLP-1R agonists, including exenatide, enable to exhibit a long-term positive impact on β -cells mass and function in human. Due to the current lack of noninvasive imaging methods that can view directly in-vivo quantitative assessment of β -cells mass in humans (Koliaki and Doupis, 2011), one of the reasonable options to test the validity of this interesting theory would be to exert long-term clinical trials to examine the durability of glycemic control that can be achieved with exenatide. In addition, since exenatide enables to hyper-activate β -cells and increase β -cell proliferation, there has been concern regarding the risk of pancreatitis and pancreatic cancer with GLP-1R agonists, exenatide. In 2 year clinical study with exenatide, there were no significant report on pancreatitis and pancreatic cancer, however, preclinical study with mice, liraglutide, one of GLP-1 analog, induced the proliferation of thyroid cancer cells (Koliaki and Doupis, 2011). Even though human thyroid expresses less GLP-1R gene than mouse thyroid cell lines and there was no significant reports on this issue in human clinical studies (Koliaki and Doupis, 2011), further studies needs to clarify these issues.

In this study, I showed that exenatide treatment significantly inhibited postprandial blood glucose excursions without risk of hypoglycemia and protected β -cells from high glucose, ER stress, and oxidative stress. However, as peptide, exenatide must be administered by injection. Thus, the orally active drugs

activating GLP-1R directly or indirectly can be a good substitute to exenatide and powerful and promising drugs to treat the patients with type 2 diabetes.

CHAPTER 2.

Drugs activating GLP-1R directly or indirectly

SECTION 1.

**Different modes of action between
Cpd.2 and Cpd.B, small GLP-1R
agonists.**

INTRODUCTION

Glucagon-like peptide-1 (GLP-1), one of the incretin hormones, is synthesized in L-cells of the small intestine and rapidly released after food intake (Drucker, 2006). GLP-1 activates its own specific receptor, GLP-1R, thereby stimulating the adenylyl cyclase pathway resulting in an increase in intracellular cAMP, Ca^{2+} influx, and insulin secretion in β -cells (Drucker, 2006; Yada et al., 1993). In patients with type 2 diabetes, GLP-1 concentration is reduced and when GLP-1 is administered, insulin secretory function is restored. The effects also include an anti-apoptotic effect in pancreatic β -cells, inhibition of gastric emptying, and lowered food intake leading to decreased body weight gain (Legakis et al., 2003; Garber et al., 2011; Nauck et al., 1993; Zander et al., 2002). Thus, GLP-1 is a critical target for new therapies for type 2 diabetes.

However, active GLP-1 is rapidly degraded by dipeptidyl peptidase-4 (DPP-4), which removes the two N-terminal GLP-1 amino acid residues and generates the biologically inactive form, GLP-1 (9–36) (Vilsboll et al., 2003). For this reason, GLP-1 has presented considerable difficulties for therapeutic use; thus, longer-acting derivatives of GLP-1, such as exenatide and liraglutide, have been developed (DeFronzo et al., 2005; Knudsen et al., 2000; Nauck et al., 2005). Exenatide, a 50% sequence homologue to native GLP-1, activates GLP-1R more potently than GLP-1 and has been approved to treat type 2 diabetes (DeFronzo et al., 2005; Montrose-Rafizadeh et al., 1997).

Although GLP-1 and its derivatives have therapeutic potential, these molecules must be administered by injection; thus, many researchers are

attempting to discover orally active and small-molecule GLP-1R agonists (Knudsen et al., 2007; Chen et al., 2007; Teng et al., 2007). Compound 2 (6,7-dichloro-2-methylsulfonyl-2-N-tert-butylaminoquinoxaline) and compound B (4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine) are small molecule ago-allosteric modulators of GLP-1R (Knudsen et al., 2007; Sloop et al., 2010; Coopman et al., 2010). Both compound 2 and compound B increase cAMP by activating GLP-1R and increase insulin secretion *in vivo* or *ex vivo*. Compound 2 increases the affinity of GLP-1 for GLP-1R; however, no reports are available for compound B (Knudsen et al., 2007; Coopman et al., 2010). Furthermore, exendin(9–39) increases the maximum efficacy of compound 2, but does not alter the compound B response (Sloop et al., 2010; Coopman et al., 2010). Coopman *et al.* reported that although compound 2 and GLP-1 have comparable GLP-1R activation effects, these molecules have different molecular signaling, such as calcium influx response and receptor internalization (Coopman et al., 2010). However, the modes of action of compound 2 and compound B have not been compared directly. In this study, I compared the modes of action of compound 2 and compound B for activating GLP-1R using CHO cells transiently expressing full-length GLP-1R or the extracellular domain truncated at the N-terminus of GLP-1R.

EXPERIMENTAL PROCEDURE

1. Materials

Compound 2 and compound B were synthesized at Dong-A Pharm. Research Center (Yong-In, South Korea). GLP-1 (GLP-1 (7–36) amide) and GLP-1 (9–36) were purchased from Tocris (St. Louis, MO, USA). Exenatide and several of its fragments (Ex3 (Ex (3–39), Ex4 (Ex (4–39), Ex5 (Ex (5–39), Ex7 (Ex (7–39), Ex9 (Ex (9–39), and Ex10 (Ex (10–39) were obtained from Anaspec (Fremont, CA, USA). The human GLP-1R expression vector (pCMV-hGLP-1R) was obtained from Origene (Rockville, MD, USA). pCRE-luciferase was obtained from Stratagene (La Jolla, CA, USA), and the pRL-TK expression vector was obtained from Promega (Madison, WI, USA).

2. Cell culture

CHO cells (Chinese hamster ovary cell line, passages 10–25) were cultured in α -MEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μ g/mL streptomycin at 37°C in a 5% CO₂-humidified incubator.

3. Transient transfection and the dual-luciferase reporter assay

CHO cells were seeded in 96-well plates at a density of 15,000 cells per well the day before transfection. Transient transfection was conducted using Lipofectamine and the Plus Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, cells were transfected with 10

ng/well pCMV6-hGLP1R, 100 ng/well pCRE-luciferase, and 10 ng/well pRL-TK, which acted as a transfection efficiency control. After 24h, the compounds were solubilized in DMSO and added to cells for 6 h. After the incubation, luciferase activity was measured using the Dual-Glo Luciferase Assay system (Promega) according to the manufacturer's protocol. Luminescence was measured by Lmax II (Molecular Devices, Sunnyvale, CA, USA). All assays were executed in triplicate, and results are presented as mean \pm standard error of the mean (SEM). Data are expressed as a percentage of maximum stimulation response induced by GLP-1.

4. Construction of the Δ -ECD-hGLP-1R plasmid

To generate the pCMV6- Δ -ECD-hGLP-1R (deletion of N-terminal 1–138 amino acids of human GLP-1R) construct, the Δ -ECD-hGLP-1R coding sequence was obtained from the pCMV6-hGLP-1R expression vector using the polymerase chain reaction (PCR) (Dyad Peltier Thermal Cycler, Bio-Rad, Hercules, CA, USA) with specific primers. The PCR product was digested with the *Sall* and *XhoI* restriction enzymes and ligated into the pCMV6 vector. The plasmid was purified and confirmed by enzyme mapping.

5. Calcium uptake assay

The calcium uptake assay was conducted using the FLIPR Calcium 5 Assay kit (R8185, Molecular Devices) in accordance with the manufacturer's instructions. Briefly, CHO cells (15,000 cells/well) were seeded in a black 96-well plate (Corning, Lowell, MA, USA), and the cells were transfected with 120 ng of pCMV6-hGLP-1R after an overnight incubation. After 24 h, the transfected cells

were loaded with 1× calcium dye in Hank's balanced salt solution (5.4 mM KCl, 4.2 mM NaHCO₃, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 0.6 mM MgSO₄, and 137 mM NaCl, pH 7.4) at 37°C. After 1 h, intracellular calcium changes were recorded at an excitation wavelength of 485 nm and an emission wavelength of 525 nm at 1.3 sec intervals for 350 sec using a Multireader Flexstation-3 (Molecular Devices). Compounds solubilized in DMSO were prepared in another 96-well plate and automatically applied to the cells after 20 sec of recording.

7. Data analysis

Data are expressed as means ± SEMs. A one-way analysis of variance using SigmaStat 2.0 (SPSS, Inc., Chicago, IL, USA) was used for comparisons among the groups, and multiple comparisons were conducted with the Student–Newman–Keuls test when significant differences were detected among groups. A $p < 0.05$ was considered significant.

RESULTS

1. Compound 2 and compound B activated hGLP-1R

I transiently expressed human GLP-1R in CHO cells and evaluated endogenous and exogenous GLP-1R agonists (GLP-1 and exenatide, respectively), an adenylyl cyclase activator (forskolin), and non-peptidic GLP-1R agonists (compound 2 and compound B). The EC₅₀ values of exenatide, GLP-1, and forskolin were 2.51 ± 0.52 pM, 34.2 ± 8.3 pM, and 398.3 ± 5.3 nM, respectively. Compounds 2 and B displayed EC₅₀ values of 1.01 ± 0.193 μ M and 4.84 ± 0.24 μ M, respectively (Fig. 14A). Compound B showed 78% efficacy relative to maximum stimulation by GLP-1, but compound 2 showed a lower efficacy of 53% (Fig 14A) because it had cytotoxic effects at higher concentrations (data not shown). The potency of forskolin in naïve CHO cells is comparable with the potency in GLP-1R expressed CHO cells; however, the other molecules did not increase luciferase activity (Fig 14B).

2. Compound 2 and compound B showed different modes of action in the presence of GLP-1, GLP-1 (9–36), or exenatide.

Compound 2 showed an additive effect on GLP-1-induced receptor activation in a concentration-dependent manner but did not increase the maximum efficacy of GLP-1 (Fig 15A). However, in contrast to compound 2, compound B increased the maximum efficacy of GLP-1 in a concentration-dependent manner (Fig 15B). Compound 2 and compound B slightly potentiated GLP-1-(9–36)-induced receptor activation, the major metabolite of GLP-1 that is generated by

DPP-4 (Fig 15A and B). In the presence of the exogenous GLP-1R agonist, exenatide, these activation patterns were identical (Fig 16A and 16B).

3. Compound 2 and compound B showed different modes of action in the presence of exenatide fragments

To further explore the different modes of action between the two compounds, I performed the reporter gene assay using several exenatide fragments. First, I checked the agonism of these peptides. As shown in Fig. 17A, the more the exenatide N-terminus was truncated, the less agonist activity was observed, and exenatide fragments truncated more than 6 aa at the N-terminus, such as Ex7, Ex9, or Ex10, did not show any increase in luciferase activity. Additionally, I assessed the antagonist effects of the various truncated exenatide peptides using a half-maximal concentration of GLP-1 (0.1 nM). Ex7, Ex9, and Ex10 showed antagonism by reducing the luciferase activity induced by GLP-1 (Fig. 17B).

Exenatide fragments that showed agonist effects, including Ex3, Ex4, and Ex5, were treated with compound 2 or compound B. Compound 2 potentiated the GLP-1R activation response of these truncated peptide agonists without increasing maximum efficacy (Fig. 18A). However, compound B increased the maximum efficacy of the peptides (Fig. 18B). Interestingly, orthosteric antagonists, such as Ex7, Ex9, and Ex10, augmented the receptor activation response of compound 2, but did not affect compound B activity (Fig. 19A and 19B).

4. Compound 2 increased the receptor activation response of compound B and was different from compound B in the calcium uptake pattern.

Next, I co-treated hGLP-1R expressed CHO cells with compound 2 and compound B. As shown in Fig. 20A, the receptor activation response of compound B was additively increased by compound 2 in a concentration-dependent manner. Moreover, when extracellular-domain truncated GLP-1R (Δ -ECD-GLP-1 receptor) was expressed, both compound 2 and compound B activated the truncated receptor, and the efficacy of compound B was also additively increased by compound 2 (Fig. 20B).

Activation of GLP-1R leads to an influx of intracellular calcium (Drucker, 2006; Yada et al., 1993). The calcium influx assay was performed in CHO cells expressing hGLP-1R to evaluate whether compound 2 and compound B have different calcium influx patterns. Compound 2 evoked a response in a concentration-dependent manner by slowly increasing calcium influx, which was longer lasting than the response evoked by GLP-1 (Fig 21A). However, compound B showed a different intracellular calcium influx pattern from that of compound 2. Compound B also slowly increased the calcium influx in a concentration-dependent manner, but the response lasted longer than that of compound 2 and GLP-1 (Fig. 21B).

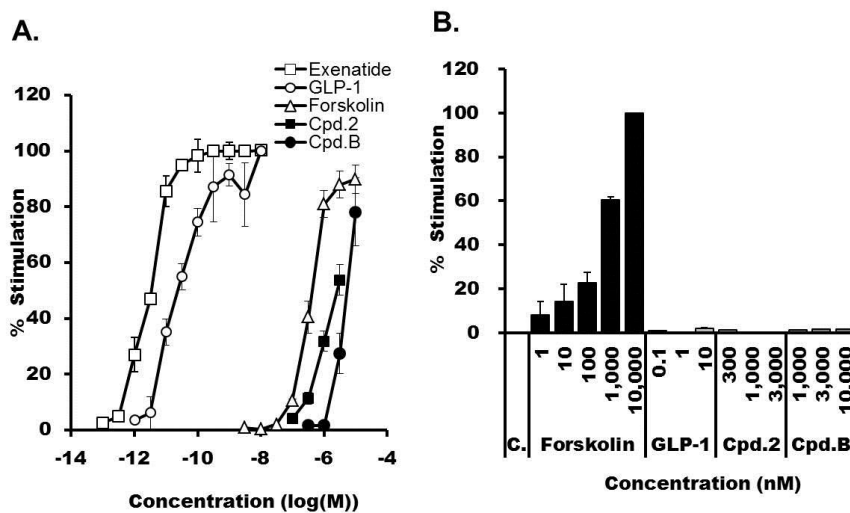


Figure 14. Potency and selectivity of two compounds for GLP-1R activation.

CHO cells transfected with hGLP-1R, pCRE-Luc, and pRL-TK (A) or pCRE-Luc and pRL-TK without hGLP-1R (B) were treated with GLP-1, forskolin, exenatide, compound 2, or compound B and luciferase activities were measured. Cpd.2, compound 2; Cpd.B, compound B. Data are expressed as a percentage of maximum stimulation response induced by GLP-1 and are mean \pm SEM.

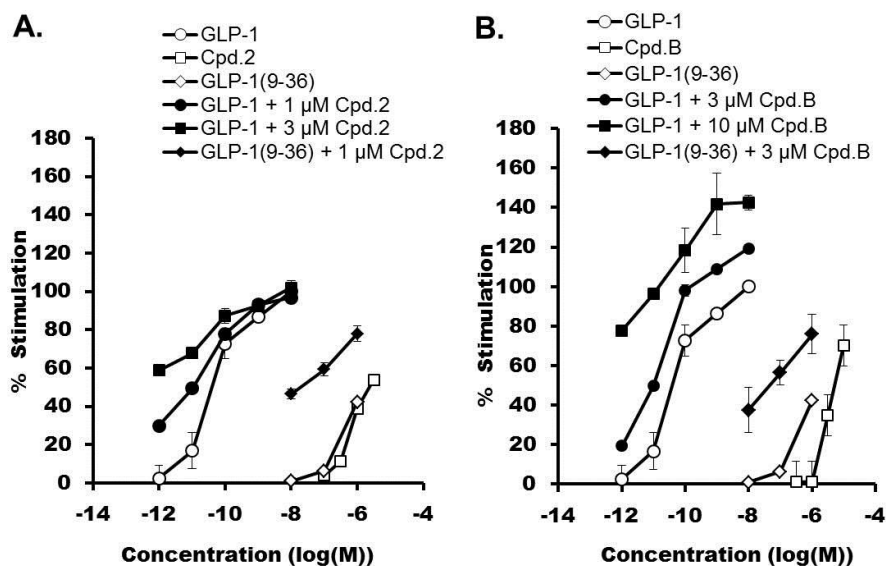


Figure 15. Different receptor activation responses of two compounds in the presence of GLP-1 or GLP-1 (9–36).

(A, B) CHO cells expressing hGLP-1R were co-treated with compound 2 or compound B with GLP-1 or GLP-1 (9–36) and luciferase activities were measured. Cpd.2, compound 2; Cpd.B, compound B. Data are expressed as a percentage of maximum stimulation response induced by GLP-1 and are mean \pm SEM.

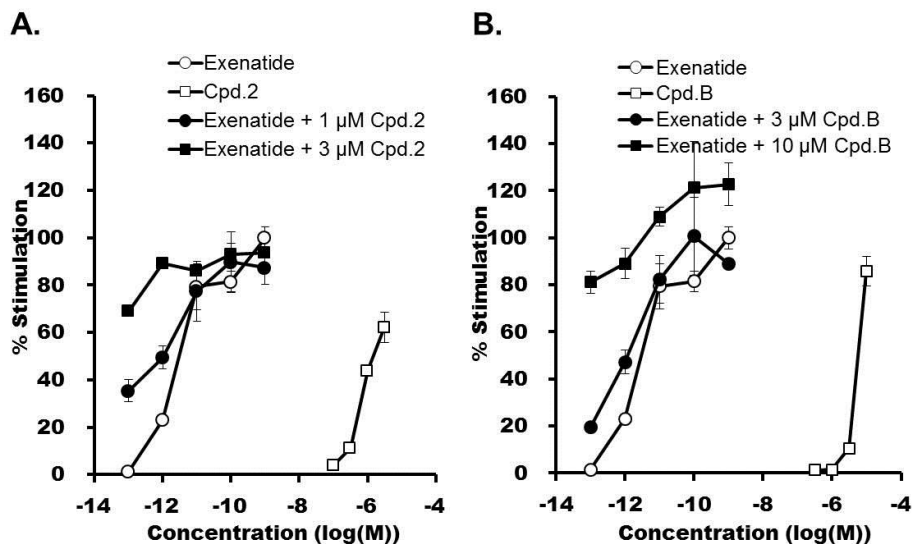


Figure 16. Different receptor activation responses of two compounds in the presence of exenatide.

(A,B) CHO cells expressing human glucagon-like peptide-1 receptor (hGLP-1R) were co-treated with compound 2 or compound B with exenatide, and luciferase activities were measured; Cpd.2, compound 2; Cpd.B, compound B. Data are expressed as a percentage of maximum stimulation response induced by GLP-1 and are mean \pm SEM

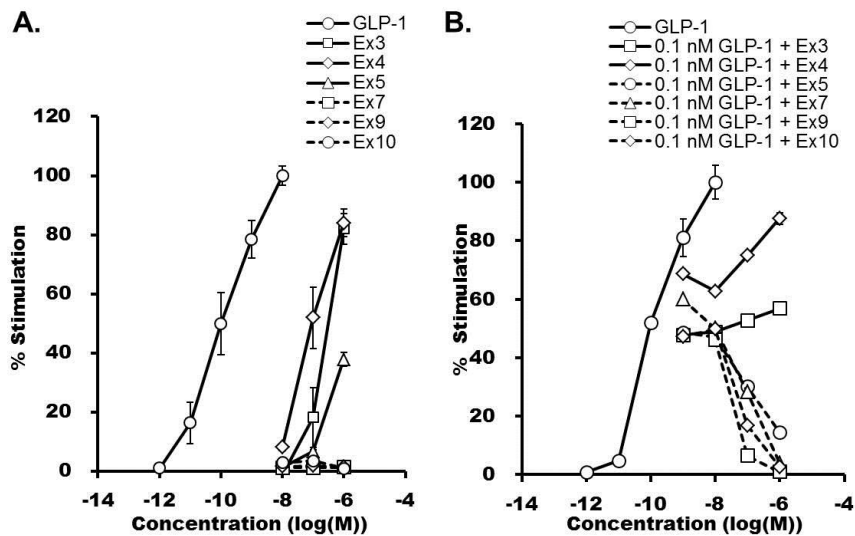


Figure 17. Evaluation of agonism or antagonism of exenatide fragments .

(A) CHO cells expressing hGLP-1R were treated with exenatide fragments (Ex3, Ex4, Ex5, Ex7, Ex9, or Ex10). (B) To evaluate the antagonism to exenatide fragments, CHO cells expressing hGLP-1R were co-treated with a half-maximal concentration of GLP-1 (0.1 nM). Ex3, Ex (3–39), indicates two N-terminal amino acids truncated on exenatide; Ex4, Ex (4–39); Ex5, Ex (5–39); Ex7, Ex (7–39); Ex9, Ex (9–39); Ex10, Ex (10–39); Data are expressed as a percentage of maximum stimulation response induced by GLP-1 and are mean \pm SEM

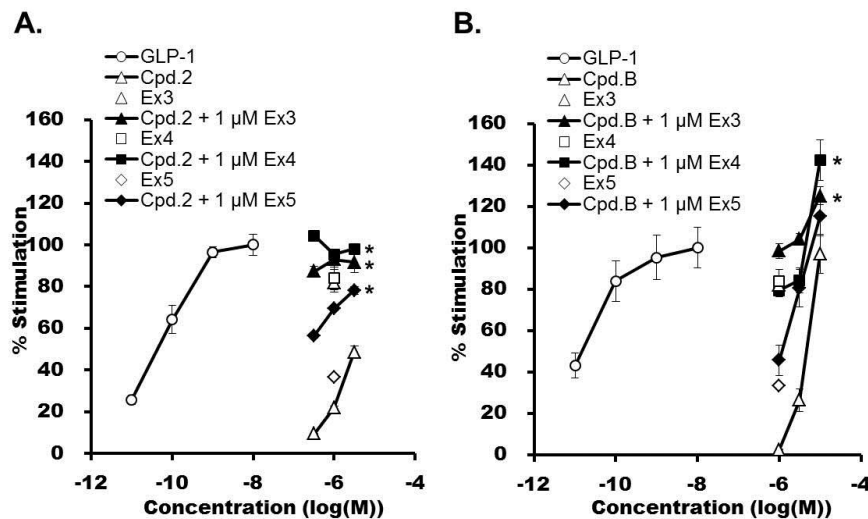


Figure 18. Different modes of action of two compounds for GLP-1R activation in the presence of Ex3, Ex4, or Ex5.

(A, B) CHO cells expressing hGLP-1R were co-treated with compound 2 or compound B and Ex3, Ex4, or Ex5. Data are expressed as a percentage of maximum stimulation response induced by GLP-1 and mean \pm SEM. * P < 0.05 vs. the receptor activation response of compound 2 or compound B.

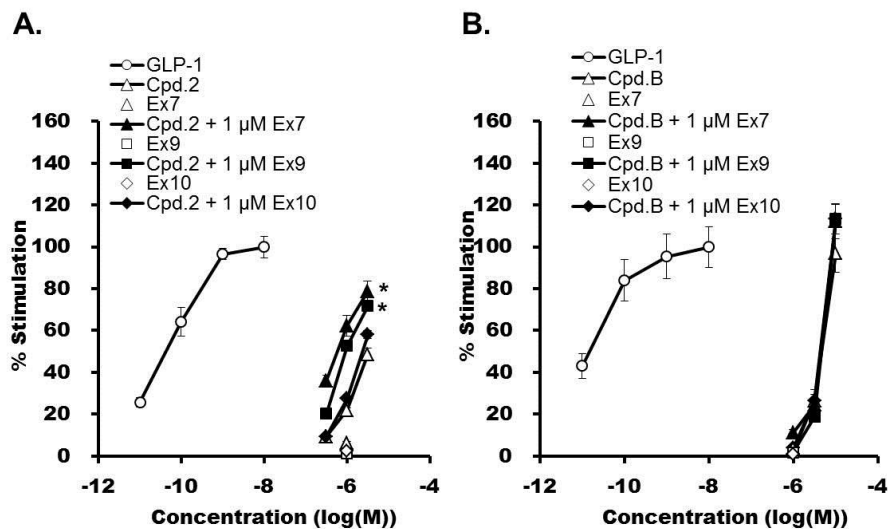


Figure 19. Different modes of action of two compounds for GLP-1R activation in the presence of Ex7, Ex9, or Ex10.

(A, B) CHO cells expressing hGLP-1R were cotreated with compound 2 or compound B and Ex7, Ex9, or Ex10. Data are expressed as a percentage of maximum stimulation response induced by GLP-1 and mean \pm SEM. $*P < 0.05$ vs. the receptor activation response of compound 2 or compound B.

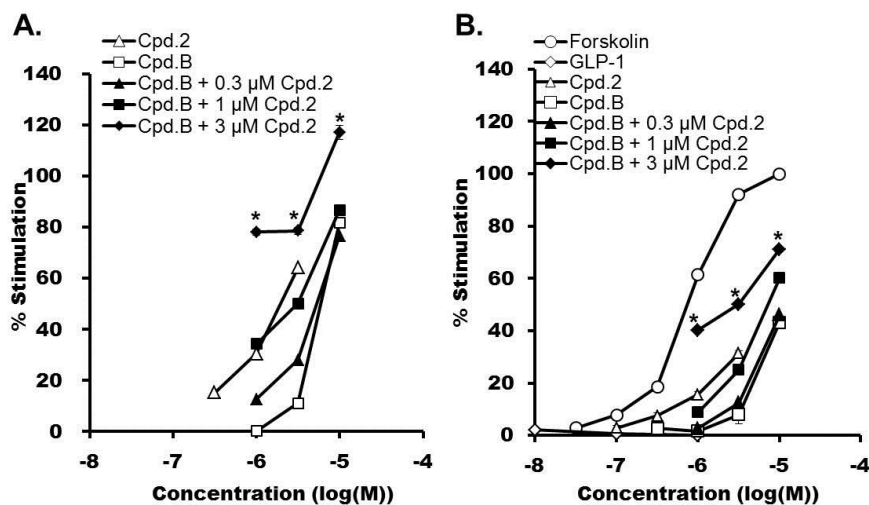


Figure 20.Co-treatment of compound 2 and compound B.

CHO cells expressing full-length hGLP-1R (A) or N-terminal extracellular domain truncated hGLP-1R (B) were co-treated with compound 2 and compound B, and luciferase activities were measured. Data are expressed as a percentage of maximum stimulation response induced by the forskolin. * $P < 0.05$ vs. the receptor activation response of compound 2 or compound B.

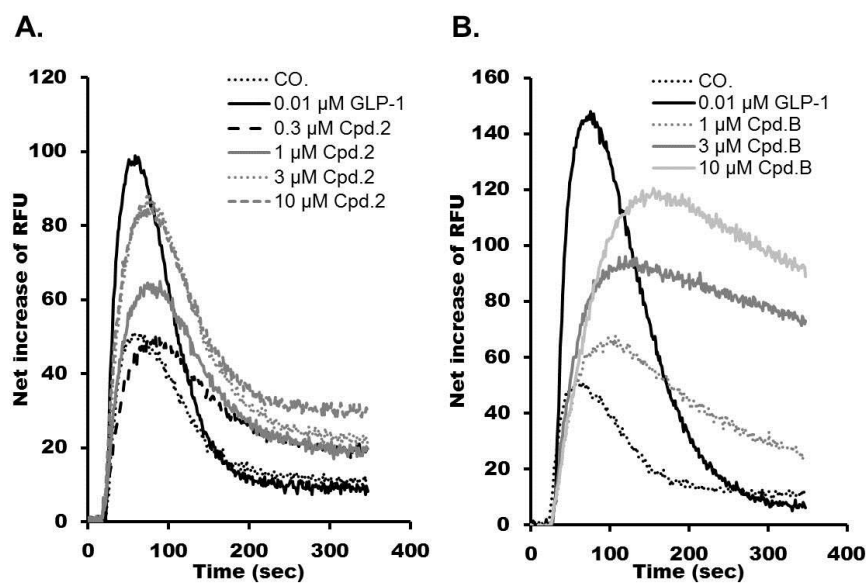


Figure 21. Different calcium influx patterns of two compounds

(A, B) Representative calcium uptake response figures for compound 2 and compound B. CHO cells were treated with each compounds, and calcium influx was measured using the Calcium 5 Assay kit.

DISCUSSION

In this study, I elucidated the different modes of action of compound 2 and compound B, which are well-known ago-allosteric modulators of GLP-1R. Compound 2 potentiated the GLP-1R and GLP-1 activation response of other full or partial orthosteric agonists without increasing maximum efficacy. However, compound B increased the efficacy of those agonists. The N-terminal amino acid of the peptide agonist binds at the GLP-1R transmembrane domain (Montrose-Rafizadeh et al., 1997). While two amino acids at the N-terminus of the peptide agonists are critical for binding and activation of the receptor, I here identified that the third to fifth amino acids of exenatide interacted with GLP-1R, and binding was potentiated by both compounds. Furthermore, three GLP-1R orthosteric antagonists augmented compound 2-induced receptor activation, but had no effect on the compound B response, similar to a previous result with exendin (9–39) (Knudsen et al., 2007; Sloop et al., 2010). Although the mechanism that caused this discrepancy is unclear, these findings suggest that small compounds binding at the allosteric site can modulate agonist and antagonist binding at an orthosteric site or vice versa, in a compound-dependent manner.

Lin *et al.* used homology modeling to discover that compound 2 binds at an allosteric site by hydrophobic interaction (Lin et al., 2009). However, there is no report on the number of allosteric GLP-1R sites. When I co-treated CHO cells transiently expressing full-length or the N-terminus extracellular domain truncated form of human GLP-1R with both compounds, receptor activation of compound B was additively increased by compound 2 at both types of GLP-1R. These results

indicate that the N-terminal domain of GLP-1R is not involved in receptor activation by the two compound agonists. If the two compounds bind the same site, maximum efficacy would be saturated, as described for other GPCR studies (Rees et al., 2002). Unfortunately, due to their low potency and/or cytotoxicity, I failed to identify the changes in maximum efficacy.

Compound 2 and GLP-1 result in different calcium influx patterns (Coopman et al., 2010). The different calcium influx pattern between compound 2 and compound B in this study might also have been caused by different conformational changes in the GLP-1R and thereby different subsequent molecular signaling might have been induced. Compound 2 and compound B may also have different responses to receptor internalization. Coopman *et al.* reported that compound 2 causes less GLP-1R internalization than that of GLP-1 (Coopman et al., 2010). In contrast, Jensen *et al.* reported that an allosteric activator can act by stimulating receptor dimerization (Jensen et al., 2004). Therefore, further studies are necessary to evaluate and compare the effect of compound 2 and compound B on GLP-1R internalization and dimerization.

Taken together, our findings suggest that although compound 2 and compound B have comparable modes of GLP-1 reactivation, they have different modes of action in the absence or presence of orthosteric agonists and antagonists. This discrepancy between the two compounds might be caused by different conformational changes in the GLP-1 receptor and subsequent cell signaling, such as the calcium influx pattern.

Section 2.

Validation of anti-diabetic effects of a novel DPP 4 inhibitor, DA-1229

Introduction

Glucagon-like peptide-1 (GLP-1) is an insulinotropic incretin hormone (Orskov et al., 1994). GLP-1 is secreted from gut endocrine L-cells by meals and nutrients. The releasing pattern of GLP-1 is biphasic: the first phase occurring at 10-15 min after oral injection and the second phase occurring at 30-60 min (Lee and Jun, 2014). Since majority of L cells are located in the distal region of the small intestine, the first secretion of GLP-1 seems to be regulated by neurotransmitters and the vagus nerve rather than by direct activation of L cells (Rocca and Brubaker, 1999). The molecular signaling for GLP-1 secretion involves glucose-sensing signals including glucokinase, ATP-dependent potassium channel, and sodium glucose cotransporter-1 (Gribble et al., 2003). GPRs (GPR119, GPR40, and GPR120), expressed in the intestine, are also involved in GLP-1 secretion (Whalley et al., 2011).

The GLP-1 (1-37) precursor peptide is modified to the major active circulating form, GLP-1 (7-36) amide, by post-translational modification in L-cells (Orskov et al., 1994). Circulating GLP-1(7-36) amide is rapidly cleaved by the DPP-4 to GLP-1 (9-36), causing a GLP-1 half-life of about 2 min. The circulating GLP-1 is finally eliminated through renal and hepatic clearance (Lee and Jun, 2014).

DPP-4 inhibitors are small molecular-weight and orally administrated drugs that inhibit > 90% of DPP-4 activity. These inhibitors prevent the rapid degradation of GLP-1, thereby improving glycemic control by preventing the rapid degradation of GLP-1 and increasing of active GLP-1 level (Brunton, 2014).

Currently, there are three Food and Drug Administration (FDA)-approved DPP-4 inhibitors: sitagliptin, saxagliptin and linagliptin.

DA-1229, (*R*)-4-[(*R*)-3-amino-4-(2,4,5-trifluorophenyl)-butanoyl]-3-(*t*-butoxymethyl)-piperazin-2-one, a novel and orally available DPP-4 inhibitor under development to improve glycemic control in type 2 diabetic patients, was synthesized in an effort to search for more potent and selective DPP-4 inhibitors (Fig. 3) (Kim et al., 2011). In this study, to ascertain its therapeutic potential, I investigated the pharmacodynamic profile of DA-1229 and effects of DA-1229 on insulin resistance and development of diabetes in HF and db/db mice.

EXPERIMENTAL PROCEDURE

1. DPP-4 enzyme inhibition assay in vitro

To measure the activity of DPP-4, fluorogenic assay was employed using Gly-Pro-AMC, which is cleaved by the enzyme to release fluorescent aminoethylcoumarin (AMC). Compounds were incubated with recombinant DPP-4 or Caco-2 lysate and 50 μ M Gly-Pro-AMC in a buffer containing 25 mM Tris/HCl, pH 8.0 (1 mg/ml bovine serum albumin was added only for recombinant human DPP-4). The assay was performed at 25°C for 1 h in a total reaction volume of 200 μ L. Liberated AMC was detected using an excitation wavelength of 360 nm and an emission wavelength of 465 nm.

2. Glucose lowering mechanism of DA-1229 in C57BL/6 mice

Six-week-old animals were acclimated for 1 week and assigned randomly to corresponding groups (6 animals per group). To measure time-dependent changes in plasma GLP-1 and insulin levels, an oral glucose tolerance test was performed, revealing peak plasma levels of GLP-1 and insulin at 5 and 10 min after glucose loading, respectively.

The mice were fasted overnight and DA-1229 (0.1~3 mg/kg; suspended in 0.5% (w/v) methyl cellulose) was administered orally 1 h before the oral glucose (2 g/kg) challenge. The plasma active GLP-1 (Linco Research, St Charles, MO), insulin (Shibayagi, Gunma, Japan) and blood glucose levels were measured at each indicated time point. The blood glucose excursions for 90 min are represented as the area under the curve ($AUC_{0-90min}$). Blood glucose AUC values of glucose-

challenged groups were normalized by subtracting that of distilled water-challenged non-glucose control and then the percent inhibition values for each treatment were generated using the normalized AUC values over the vehicle-treated glucose control. The plasma DPP-4 activity was measured at 5 min after the glucose loading, as described by Kim et al. (Kim et al., 2005).

3. Antidiabetic effects in high fat diet-fed (HF) mice

At 6 weeks of age, the C57BL/6J mice were fed a high fat diet (20% protein, 35.5% fat and 36.3% carbohydrate as weight per weight; Research Diets, New Brunswick, NJ) ad libitum for 24 weeks and maintained at 21°C on a 12:12-h light-dark cycle. Male HF mice (30 weeks of age) were housed 6 per group and were divided evenly according to blood glucose levels and body weight. Each group of mice was then treated with a DA-1229 diet admixture (0.05% (w/w) and 0.2% (w/w); approximately 30 mg/kg/day and 100 mg/kg/day, respectively) for 8 weeks. Non-fasting glucose levels, body weight and diet consumption were measured weekly.

An oral glucose tolerance test was conducted after 7 weeks of treatment with DA-1229 as a diet admixture. The mice were fasted for 4 h and the basal blood glucose levels ($t = -30$ min) were measured from the tail vein blood using a glucometer (Roche, AccuChek Active, Ireland). The mice were then challenged orally with a glucose solution (2 g/10 ml/kg) and blood glucose levels were measured from the tail vein at the indicated time points for 2 h after the glucose challenge. The blood glucose excursions for 2 h are represented as the area under the curve (AUC_{0-2h}).

After the 8-week treatment, the mice were fasted for 4 h and blood was collected from the orbital venous sinus in a heparin-filled tube. The plasma was separated by centrifugation at 5,000 x g for 5 min. The plasma glucose levels were measured directly by an enzymatic method using a Konelab 20i[®] analyzer (Waltham, MA), and the plasma insulin was measured using insulin ELISA kits.

4. Effect on the onset of diabetes in young db/db mice

Five-week-old db/db mice were acclimated for 1 week and allocated to corresponding groups (8 animals per group). The mice were treated with DA-1229 as a diet admixture (0.3% (w/w); approximately 300 mg/kg/day) or normal chow for 4 weeks. The change in HbA1c levels was measured with DCA 2000⁺ (Bayer Healthcare, Wuppertal, Germany). After sacrifice, the plasma insulin levels were measured. The whole pancreas was then isolated immediately free from fat and other non-pancreatic tissues. After measuring the wet weight, the isolated pancreas was frozen in liquid nitrogen and stored at -70°C until needed.

5. Data analysis

The data was expressed as the mean \pm S.E.M. Statistical analyses were performed using SigmaStat 2.0 (SPSS, Chicago, IL). Two-group comparisons were performed using a Student's t-test. For multi-group comparisons, when ANOVA indicated significant differences among the groups ($P < 0.05$), the differences were evaluated further using a Student-Newman-Keuls multiple comparison test. P values under 0.05 were considered significant.

RESULTS

1. Potency and selectivity of DA-1229

DA-1229 effectively inhibited the soluble form of both human and mouse DPP-4 with IC₅₀ values of 0.98 nM and 3.59 nM, respectively (Fig. 22). There was little species difference in the inhibitory potency. For membrane-bound DPP-4 isolated from Caco-2 cells, DA-1229 also exhibited potent inhibition with an IC₅₀ of 1.26 nM (Fig. 1B). As shown in figure 1, DA-1229 showed excellent selectivity to DPP-4 compared to human DPP8 and DPP9 with a 6,000 times higher potency (Fig. 22).

2. The mechanism of DA-1229 on plasma glucose lowering in C57BL/6 mice

The oral glucose tolerance test in C57BL/6 mice showed that the DA-1229 treatment significantly inhibited postprandial blood glucose excursions in a dose-dependent manner (Fig. 23A). One hour after administration, the DA-1229 treatment inhibited plasma DPP-4 activity in a dose-dependent manner (Fig. 23B). In proportion to the degree of DPP-4 inhibition, peak plasma levels of GLP-1 and insulin increased, in contrast to vehicle treated controls (Fig. 23C). Therefore, these results suggest plasma DPP-4 inhibition by DA-1229 reduces the level of endogenous GLP-1 degradation, causing an increase in plasma GLP-1 levels, which would stimulate pancreatic insulin secretion (data not shown), and ultimately reduce blood glucose excursions.

3. Insulin-sensitizing effects in HF mice

An 8-week study was performed to examine the chronic effects of DA-1229 on insulin sensitivity. An oral glucose tolerance test was performed at 7 weeks after treatment to assess its ability to improve glucose intolerance. After oral glucose loading, blood glucose levels were noted to be significantly higher in the HF mice than the lean mice. The DA-1229 treatment resulted in a dose-dependent improvement in postprandial blood glucose excursions (29% and 69% decrease in the AUC_{0-2h}, respectively) (Fig. 24A).

At the end of the study period, fasting plasma glucose levels were lower in the DA-1229 treated groups than the vehicle-treated control (data not shown). The DA-1229 treatment resulted in significant decreases in the plasma insulin levels increased by a high fat diet (Fig. 24B), as well as a dose-dependent improvement in the HOMA-IR compared to the vehicle-treated control (Fig. 24C).

4. Effects on the onset of diabetes in young db/db mice

To examine the effects on the development of diabetes, DA-1229, as a diet admixture (0.3% (w/w); approximately 300 mg/kg/day) was administered to 6-week-old db/db mice for 4 weeks. The DA-1229 treatment produced more than 95% inhibition of the plasma DPP-4 activity (data not shown). At the end of the study period, the blood glucose levels of the vehicle-treated db/db mice were higher than the lean mice. DA-1229 significantly inhibited the elevation of blood glucose for the first 2 weeks compared to the db/db control (Fig. 25A). After 4 weeks of treatment, the glycated hemoglobin concentration (HbA1c, %) of the db/db control was 2.4% higher than the baseline level. In the DA-1229 treated group, the HbA1c level was significantly lower than the db/db control (Fig. 25B). This reflects

lowered blood glucose levels in the early stages. DA-1229 treatment caused a significant increase in the pancreatic insulin content (2.3 fold) compared to the db/db control (Fig. 25C). This suggested that although there was no change in the levels of plasma insulin, the insulin-producing potential remained higher than the vehicle treated control.

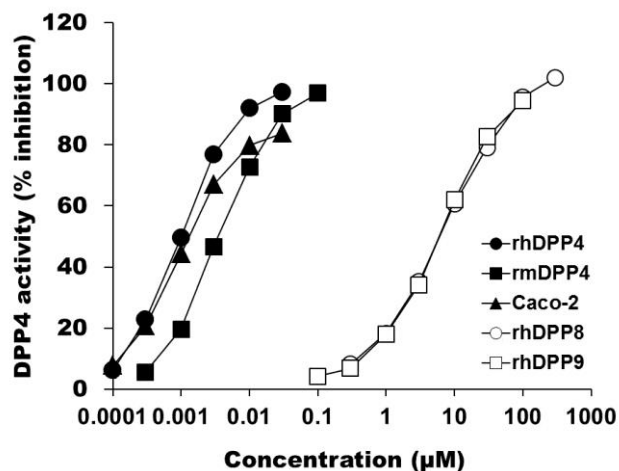


Figure 22. The potency and selectivity of DA-1229.

Recombinant enzymes were pre-incubated with DA-1229. The reaction was initiated by adding fluorogenic substrates, such as Gly-Pro-AMC for DPP-4 and DPP9 or Ala-Pro-AFC for DPP8. The data is reported as the mean \pm S.E.M. (N=2-4). The symbols are as follows; ● recombinant human DPP-4, ■ recombinant mouse DPP-4, ▲ membrane bound DPP-4, ○ recombinant human DPP8, □ recombinant human DPP9.

(This work was done in collaboration with Ha-Dong Kim)

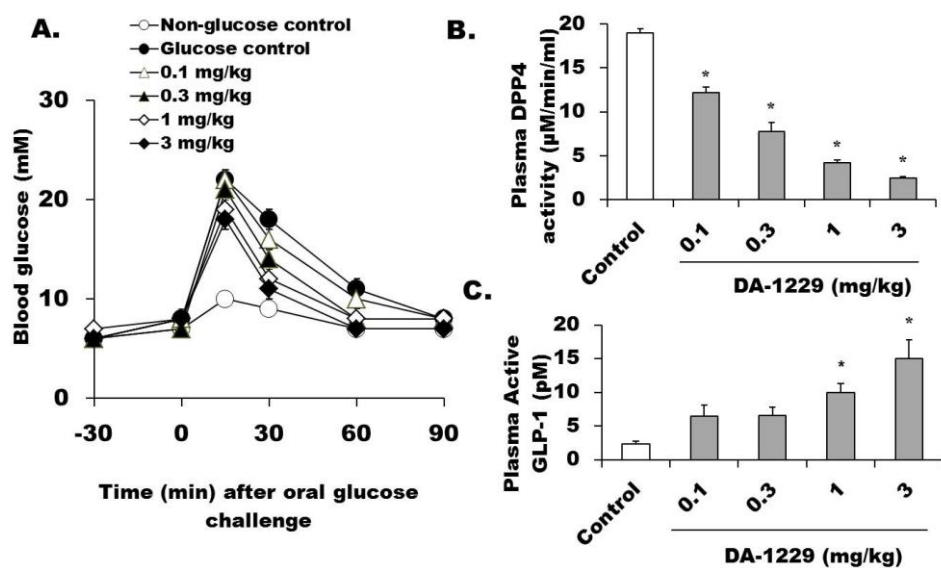


Figure 23. Effect of DA-1229 on the blood glucose excursion in C57BL/6 mice.

At 1 h post-dose, the mice were given a glucose solution (2 g/kg). After the glucose loading, blood glucose was measured for 2 h (A). The plasma DPP-4 activity (B) and peak plasma levels of GLP-1 (C) were measured at 5 min after the glucose loading. The data is reported as the mean \pm S.E.M. *, $P < 0.05$ vs. control (N=6/group).

(This work was done in collaboration with Yu Na Chae, Eun Kyoung Yang, Song-Hyen Choi, and Chang Yell Shin)

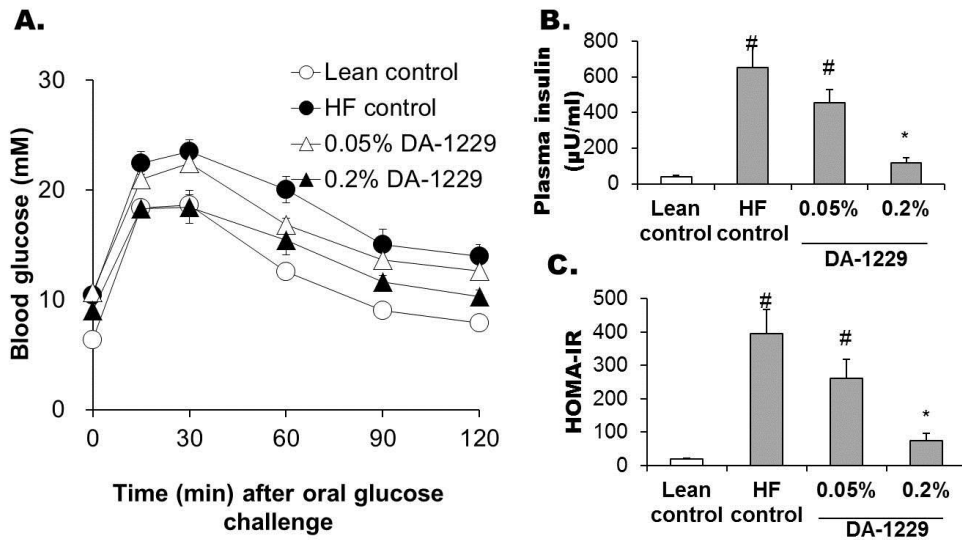


Figure 24. Insulin-sensitizing effects of DA-1229 in HF mice.

Six-week-old C57BL/6J mice were fed a high-fat diet ad libitum for 24 weeks. After that, the mice were treated with the DA-1229 diet admixture (0.05% (w/w) and 0.2% (w/w)) for 8 weeks. At week 7, the mice were fasted for 6 h prior to an oral glucose challenge (2 g/kg). The mice were then challenged orally with the glucose solution (2 g/10 ml/kg) and the blood glucose levels were measured from tail vein (A). At week 8, the animals were sacrificed, and plasma insulin levels (B) were measured. HOMA-IR was calculated (C). The data is reported as the mean \pm S.E.M. #, $P < 0.05$ HF control vs. lean control; *, $P < 0.05$ vs. HF control.

(This work was done in collaboration with Yu Na Chae, Eun Kyoung Yang, Song-Hyen Choi, and Chang Yell Shin)

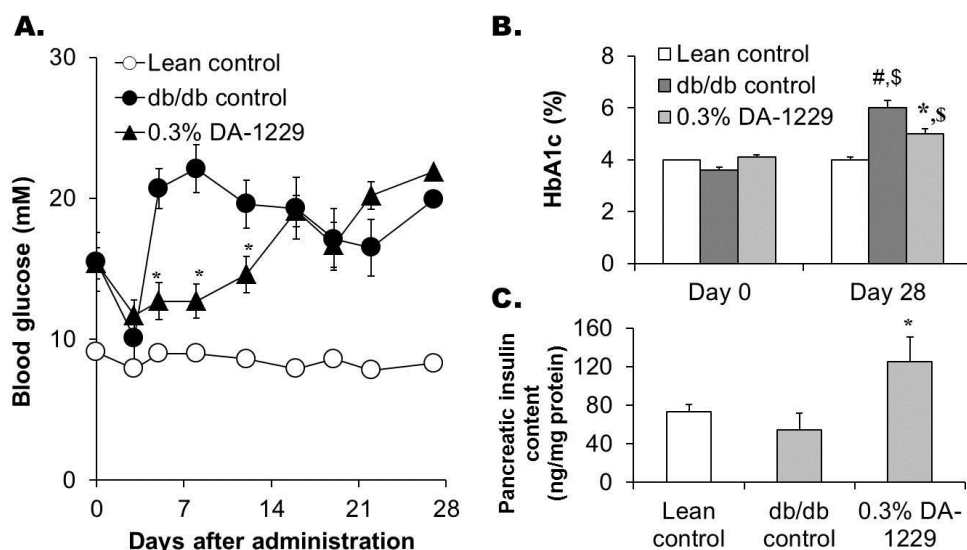


Figure 25. Effects of DA-1229 on onset delay of diabetes in db/db mice.

Six-week-old db/db mice were treated with DA-1229 as a 0.3% (w/w) diet admixture for 4 weeks. (A) DA-1229 significantly inhibited the elevation of blood glucose for the first 2 weeks compared to the db/db control. (B) After 4 weeks treatment, the DA-1229 treatment significantly suppressed the increase in HbA1c (%) compared to the db/db control (C) the pancreatic insulin content in the DA-1229 treated group was significantly higher than the db/db control. The data is reported as the mean \pm S.E.M. #, $P < 0.05$ db/db control vs. lean control at day 28; *, $P < 0.05$ vs. db/db control at day 28; \$, $P < 0.05$ vs. day 0 (N=8/group).

(This work was done in collaboration with Yu Na Chae, Eun Kyoung Yang, Song-Hyen Choi, and Chang Yell Shin)

Discussion

In this study, DA-1229, a novel DPP-4 inhibitor, was shown to be potent and selective inhibitor. Minimal or absent selectivity against closely-related enzymes to DPP-4 is necessary to reduce adverse drug reactions or toxicity. Two enzymes, DPP8 and DPP9, are closely related to DPP-4 with postproline cleavage activity (Abbott et al., 2000; Olsen and Wagtmann, 2002), and acute toxicity was reported for a nonselective inhibitor with strong inhibition of DPP8 and DPP9 (Lankas et al., 2005). Therefore, the selectivity profiles against human DPP8 and DPP9 should be established for safety concerns. DA-1229 was shown to be more than 6,000-fold selective to DPP-4 than DPP8 and DPP9. Its selectivity profile minimizes the potential risk of adverse events caused by DPP8/DPP9 inhibition.

DPP-4 inhibitors were previously reported to cause a significant decrease in glucose excursions concomitant with elevations in plasma insulin and active GLP-1 levels in normal and diabetic animals (Lee et al., 2008). Consistent with previous results, after an oral glucose challenge and DA-1229 treatment, GLP-1 levels increased, followed by an increase in insulin levels and reduced blood glucose excursions in normal mice.

Based on the hypothesis that incretin receptor activation is required for optimal glucose control (Lamont and Drucker, 2008), I evaluated its ability to improve the glucose intolerance in HF mice. In this study, HF mice exhibited impaired glucose tolerance in response to a glucose challenge, which is consistent with findings observed in type 2 diabetes patients (Lamont and Drucker, 2008). By reducing blood glucose excursions, DA-1229 improved glucose tolerance in

insulin-resistant mice. Although improvements in glucose tolerance could be attributed to weight and fat reduction as well as increased GLP-1 levels, the relative contributions of each of these factors could not be determined in the present study. A decrease in GLP-1 secretion has been observed in obese and diabetic subjects, which is related to increased plasma DPP-4 activity (Kirino et al., 2009; Lugari et al., 2004) and an impaired incretin effect (Muscelli et al., 2008; Naslund et al., 1998). A DPP-4 inhibitor can enhance the plasma levels of active GLP-1, which leads to improved β -cells/ α cell sensitivity to increase the level of glucose-dependent insulin secretion and reduced inappropriate glucagon secretion. These islet effects are associated with increased insulin sensitivity (Ahren and Foley, 2008). The homeostatic model assessment (HOMA) is a method used to quantify insulin resistance and β -cell function, correlating well with the hyperinsulinemic-euglycemic clamp method, a gold standard for determining and quantifying insulin sensitivity (DeFronzo et al., 1979; Matthews et al., 1985).

In the present study, while the DA-1229 treatment delayed the rise of blood glucose within the first two weeks of administration, DA-1229 didn't control the elevated blood glucose later, which suggests that progression of insulin resistance might be the cause of lack of efficacy in db/db mice as previously described (Nagakura et al., 2003). DA-1229 treatment also preserved the pancreatic insulin content in db/db mice, which is consistent with that of the GLP-1 agonist, exenatide (Wang and Brubaker, 2002). These findings suggest that a DPP-4 inhibitor can delay the onset of diabetes in the early stages by preventing the progression from impaired glucose tolerance to diabetes as previously reported (Sudre et al., 2002).

In summary, DA-1229 is a potent, novel and selective DPP-4 inhibitor that improves insulin sensitivity in HF mice and delays the onset of diabetes in young db/db mice. These results suggest that DA-1229 can be a new pharmacological choice for preventing and treating type 2 diabetes. Clinical studies to examine the efficacy and safety of DA-1229 in patients with type 2 diabetes are currently underway.

CHAPTER 4.

CONCLUSION

CONCLUSION

In this study, I focused on the glucagon-like peptide-1 receptor (GLP-1R.). GLP-1R signaling is well known for controlling postprandial blood glucose excursion, and some medicines activating GLP-1R directly or indirectly, such as exenatide, liraglutide, and sitagliptin, have already been approved by the FDA. However, many questions about GLP-1R signaling remain.

I. In chapter 2, the results of this study demonstrated that glucose fluctuations triggered a reduction in GLP-1R expression and an increase in ER stress. Consistently, these molecular changes subsequently resulted in impaired GLP-1 signaling in INS-1 cells, contributing to reductions in insulin secretion by GLP-1 to a greater degree than is observed in chronic hyperglycemia. From a clinical standpoint, this study suggests that strict regulation of blood glucose concentrations is necessary to prevent β -cell dysfunction, mediated by a reduction in GLP-1R expression and, subsequently, GLP-1 signaling. Exenatide, a GLP-1R agonist, decreased postprandial glucose excursions effectively without causing hypoglycemia in normal mice and prevented β -cell death induced by high glucose and ER stress in INS-1 cells. Thus, these results suggest that exenatide may be a promising medicine for treating type 2 diabetes, lowering glucose fluctuation levels and β -cell death induced by high glucose and ER stress.

II. In chapter 3, I examined the mode of actions and the anti-diabetic effects of small-molecule GLP-1R agonists and DA-1229, a novel DPP-4 inhibitor. First, I compared two orally active GLP-1R agonists: compounds 2 and B. The

results suggest that although compounds 2 and B have comparable degrees of GLP-1 activation, they have different modes of action in the absence or presence of orthosteric agonists and antagonists, indicating that these compounds likely bind different sites in GLP-1R. These data may be useful in designing and discovering novel GLP-1R agonists, and also suggest that GLP-1R has, at least, two binding sites for small GLP-1R agonists. Second, I showed that DA-1229 was a potent, novel, and selective DPP-4 inhibitor that improved insulin sensitivity in HF mice and delayed the onset of diabetes in young db/db mice. These results suggest that DA-1229 may be a new pharmacological choice for preventing and treating type 2 diabetes. Given these positive preclinical data, DA-1229 is currently in clinical development.

III. This study has several limitations and presents new questions. First, glucose fluctuation may affect many signaling pathways and the expression of signaling molecules. However, this study focused on only GLP-1R expression and signaling. To examine whole genome changes under these conditions may also provide valuable information. Second, the molecular mechanisms that result in decreased GLP-1R expression were not explained in this study. According to recent studies, hyperglycemia induces an increase in ER stress and a reduction of GLP-1R expression. Although there is no direct evidence linking ER stress and GLP-1R expression, because ER stress leads to the impairment of expression of many genes, GLP-1R expression may also be affected by ER stress. However, further studies are required to determine this. Third, I only showed the different modes of action between compounds 2 and B in an *in vitro* assay. Because the stability of these compounds in blood is not high, it is difficult to examine these compounds in

animal models. However, to assess the molecules completely, *in vivo* experiments need to be done.

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국문초록

당뇨병은 전 세계적로 위협하는 질환 중 하나이다. 가장 일반적인 당뇨병의 유형으로는 제1형 당뇨병과 제 2형 당뇨병이 있으며 전체 당뇨병 중 90-95%를 제 2형 당뇨병이 차지한다. 지속적인 혈당변동 현상은 당뇨병 환자에서 자주 관찰되는 현상이다. 일반적으로 제 2형 당뇨병 환자에서 혈당변동 현상은 크게 중요하게 여겨지지 않았다. 하지만, 최근 연구에서 지속적인 고혈당보다 혈당변동이 베타세포의 기능을 좀 더 악화시키고 당뇨병성 심혈관 합병증 발병을 증가시킨다는 보고된 바 있어 평균혈당만큼 그 중요성이 부각되고 있다. Glucagon like peptide-1 receptor (GLP-1R) 신호전달은 식후 인슐린 분비의 60%를 차지하는 등 매우 중요한 역할을 담당한다. 하지만 아직까지 혈당변동 현상이 GLP-1R 신호전달에 미치는 영향은 연구된 바가 없다.

이 연구에서는 지속적인 고혈당 현상에서 보다 혈당변동 현상이 베타세포에서 GLP-1을 매개한 분자신호전달에 좀 더 안 좋은 영향을 미칠 수 있음을 가설로 정하였다. 이 가설을 검증하기 위해 INS-1 세포주에 혈당변동을 모방할 수 있는 intermittent high glucose (IHG)와 지속적인 고혈당을 대표할 수 있는 sustained high glucose (SHG)를 72시간 동안 처리하였다. 실제 INS-1 세포주에 IHG를 처리하였을 때, SHG를 처리하였을 때에 보다 인슐린분비가 더 많이 감소되어 있음을 알 수 있었다. 같은 세포주에 IHG를 처리하였을 때, SHG 조건에서 보다 ER stress와 세포사멸 분자적 변화가 더 많이 유도되었다. 그리고 흥미롭게도 GLP-1R의 발현도 역시 더 많이 감소하였다. 이 결과로 혈당변동 현상이 ER stress의 증가와 GLP-1R의 발현을 감소시키고 그로 인해 더 많은 GLP-1R 신호전달에 안 좋은 영향을 미쳤음을 확인하였다. 이 결과는

또한, 실제 제2형 당뇨병 환자에게서 혈당조절의 중요성을 설명해 주고 있다.

다음으로, GLP-1R agonists인 exenatide가 실제 식후혈당증가를 억제하고 베타세포 사멸을 억제하는 역할이 있는지를 직접 평가하고자 하였다. INS-1 세포주에 exenatide를 처리했을 때 고 농도의 글루코스에 의한 세포사멸, 그리고 혈당변동으로 더 많이 유도된다고 알려진 oxidative stress, ER stress로 인한 세포사멸이 억제되었다. 또한 동물실험에서, exenatide가 저혈당을 유발하지 않으면서 식후혈당 증가를 효과적으로 낮추었다. 이 결과로, GLP-1R agonists가 혈당변동을 낮추면서 혈당변동으로 유도될 수 있는 ER stress와 oxidative stress를 억제할 수 있는 좋은 치료제가 될 수 있음을 확인하였다.

비록 exenatide가 좋은 당뇨치료제로의 특성을 가지고 있지만 하루에 두 번씩 피하주사로 투여해야 하는 번거로움이 있기 때문에 GLP-1R를 활성화시킬 수 있는 다른 방법에 대한 연구가 활발하게 진행 중이다. 이제까지 경구투여가 가능한 GLP-1R agonist 개발은 성공하지 못 했으나 최근 세계적인 제약 회사에서 작은 분자량으로 경구투여가 가능한, compound 2와 compound B 라고 명명한 GLP-1R agonist를 보고하여 작은 분자량의 GLP-1R agonist 개발 가능성을 제시하였다. 이 두 물질은 상이한 분자구조를 가지고 있기 때문에 서로 다른 방법으로 GLP-1R를 활성화시킬 가능성이 있으나 실제 두 물질의 작용기전에 대해서는 직접적으로 비교된 바가 없다. 이에 이 두 물질의 서로 다른 작용기전에 대한 가능성을 검증하기 위해 먼저 GLP-1R가 과발현된 CHO 세포주에 compound 2와 compound B를 잘 알려진 펩타이드 agonists와 antagonists와 같이 처리하여 그 반응을 평가하였다. 평가 결과 3종류의 반응에서 두 물질이 서로 다른 활성을 나타냄을 확인하였다. 먼저, compound 2는 내인성 또는 외인성 agonists의 반응을 증가시키지만 최대반응성은 증가시키지 않았다. 하지만, compound B는 최대반응성도 같이 증가시켰다. 두 번째로,

compound 2는 antagonists의 활성을 오히려 증가시켰으나, compound B는 antagonists와 같이 처리했을 때 아무런 활성변화도 관찰되지 않았다. 마지막으로, compound 2는 칼슘 흡수를 지속적으로 증가시킨 반면 compound B는 그렇지 않았다. 그리고 흥미롭게도 compound 2와 compound B를 같이 처리했을 때에 가산적으로 반응이 증가되었고 이 결과들로 두 물질이 GLP-1R의 서로 다른 곳에 결합할 가능성이 있음을 설명해주고 있다. 이 실험결과들을 토대로, 비록 두 개의 agonists가 유사한 GLP-1R 활성화능을 가지고 있지만 서로 다른 곳에 결합해서 서로 다른 작용 기전을 나타낼 수 있음을 알 수 있었다. 이 결과들은 또한 아직 구조가 밝혀지지 않은 GLP-1R의 구조를 이해하고 추후 GLP-1R agonist를 개발하는데 큰 도움을 줄 수 있다고 생각된다.

마지막으로 새로운 DPP-4 억제제인 DA-1229의 항당뇨효과를 평가하는 실험을 진행하였다. 평가 결과 세포 외 실험에서 DA-1229는 우수한 선택성과 활성을 나타냈다. 동물실험에서는 DA-1229가 혈 중 GLP-1과 인슐린 분비를 증가시키고 실제 식후혈당증가를 충분히 낮췄다. 추가적으로, 당뇨 병태모델인 HF-DIO 설치류모델에서의 인슐린 민감도를 증가시키고 또한 db/db 동물모델에서 당뇨 발생을 늦춤을 확인할 수 있었다. 이 상의 결과로 경구투여가 가능하면서 간접적으로 GLP-1R를 활성화시키는 DPP 4 억제제인 DA-1229가 실제 제 2형 당뇨 치료제의 가능성이 있음을 확인하였다. 이 전임상 결과로, DA-1229는 임상 시험에 진행할 수 있었고 현재 임상 3상 시험 중이다.

주요어: 제 2형 당뇨병, 혈당변동, GLP-1R, exenatide, GLP-1R agonist, DPP - 4 억제제